PHOSPHOLIPID MODIFYING ENZYMES AND THE REGULATION OF GOLGI COMPLEX STRUCTURE AND FUNCTION

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PHOSPHOLIPID MODIFYING ENZYMES AND THE REGULATION OF GOLGI COMPLEX STRUCTURE AND FUNCTION

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The secretory pathway in cells is critical for the precise synthesis, modification and directed transport of proteins, lipids, and carbohydrates. Phospholipid metabolizing enzymes are thought to contribute to the transport of cargo within the cell by altering the lipid composition of membranes and allowing them to form transport carriers including vesicles and membrane tubules. The specific phospholipid-modifying enzymes have not been fully discovered, but evidence suggests roles for both lysophospholipid acyltransferases (LPATs) and phospholipase A_2 (PLA_2) enzymes in mediating membrane trafficking.

Using inhibitor-based studies, PLA_2 activity has been shown to be important for Golgi trafficking and structure. PLA_2 antagonists block the formation of membrane tubules from the Golgi. Conversely, LPAT inhibitors cause the formation of Golgi membrane tubules. From those observations, we hypothesize a role for PLA_2 enzymes in generating membrane tubules and a role for LPATs in the negative regulation of membrane tubules, which are important for Golgi structure and function.

In this study I explore the functions of a novel protein, lysophosphatidic acid acyltransferase 3 (LPAAT3), in phospholipid metabolism, maintaining Golgi structure, membrane trafficking, and membrane tubule regulation. Using

biochemical approaches, I have shown that LPAAT3 generates phosphatidic acid in Golgi membranes. LPAAT3's activity is critical for maintaining a compact Golgi structure. Furthermore, LPAAT3 expression alters both retrograde and anterograde trafficking by regulating the formation of membranes tubules from the Golgi.

The role of PLA_2 enzymes in trafficking was also explored using inhibitors to study trafficking from the Trans Golgi Network (TGN) to the cell surface. PLA_2 inhibitors blocked trafficking to the plasma membrane and caused the accumulation of cargo in the TGN. Furthermore, the formation of TGN tubules, whose fission is regulated by protein kinase D (PKD), is dependent on PLA_2 activity as well.

I show that both LPATs and PLA_2s are important factors in regulating membrane trafficking within cells. They do this by altering the phospholipids within membranes and by promoting or regulating the formation of membrane vesicles and membrane tubules. These results contribute to our understanding of membrane trafficking and the phospholipids involved in those pathways.

BIOGRAPHICAL SKETCH

John Andrew Schmidt was born and raised in Haddonfield, NJ to Ken and Edna Schmidt with his younger brother Scott. He attended Haddonfield Memorial High School from which he graduated in 1998. During school he was active in community service organizations including the Boy Scouts where he earned the rank of Eagle. John attended Colgate University in the rural village of Hamilton in upstate New York. During college he was active in environmental causes and outdoor activities and studied public health for a semester at the University of Wales, Cardiff. He graduated in 2002 *cum laude* with a degree in molecular biology. During college his research included using molecular genetics to understand plant-insect coevolution. At the Coriell Institute for Medical Research he used cytogenetics to examine the disease ataxia telangiectasia and cancer. In 2002 John started graduate school at Cornell University in Ithaca, New York working towards a doctoral degree in biochemistry, molecular, and cell biology in the department of Molecular Biology and Genetics. He joined the lab of Bill Brown to study membrane trafficking. His hobbies include talking politics, visiting art galleries, wine tasting, playing with his dog Ziggy, and brewing beer. John will be doing post-doctoral research in the lab of Wei Guo at the University of Pennsylvania in Philadelphia.

This dissertation is dedicated to my family.

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I am very thankful to have such a supporting family, which has helped me get through college and graduate school without becoming hungry or homeless. I also thank Cory Walker for his encouragement and patience over the past four years.

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LIST OF ABBREVIATIONS

ACAT	acyl-coenzyme A: cholesterol acyltransferase
ADP	adenosine diphosphate
AGPAT	1-acyl *sn*-glycerol-3-phosphate *sn*-2-acyltransferase
AP	adaptor protein
ARF1	ADP-ribosylation factor 1
BBC	bovine brain cytosol
BEL	bromoenol lactone
BFA	brefeldin A
BSA	bovine serum albumin
CI-976	2,2-dimethyl-N-(2,4,6-trimethyoxyphenyl) docecanamide
CoA	coenzyme A
COPI	coat protein I
COPII	coat protein II
BARS	BFA-ADP-ribosylated substrate
DMSO	dimethyl sulfoxide
EM	electron microscopy
ER	endoplasmic reticulum
ERGIC	ER-Golgi-intermediate compartment
FITC	fluorescein isothiocyanate
Gal T	galactosyltransferase

GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GPP130	Golgi phosphoprotein 130 kD
IC50	concentration required for 50% inhibition
LPA	lysophosphatidic acid
LPA-AT	lysophosphatidic acid acyltransferase
LPAAT	lysophospholipid acyltransferase
LPC	lysophosphatidylcholine
LPC-AT	lysophosphatidylcholine acyltransferase
LPE	lysophosphatidylethanolamine
LPE-AT	lysophosphatidylethanolamine acyltransferase
LPS	lysophosphatidylserine
LPS-AT	lysophosphatidylserine acyltransferase
LPL	lysophospholipid
Mann II	mannosidase II
M6PR	mannose-6-phosphate receptor
MEM	minimal essential media
MTOC	microtubule organizing center
ONO-RS-082	2-(*p*-amylcinnamoyl) amino-4 chlorobenzoic acid
PA	phosphatidic acid
PBS	phosphate buffered saline
PDI	protein disulfide isomerase
PKD	protein kinase D

PKD-KD	kinase dead protein kinase D
PL	phospholipid
PLA_2	phospholipase A_2
PNS	post-nuclear supernatant
TGN	trans Golgi network
TLC	thin layer chromatography
TRITC	trimethyl rhodamine isothiocyanate
VSV-G ts045	vesicular stomatitis virus G-protein temperature sensitive

CHAPTER 1

General Introduction

All living cells require a process by which newly synthesized proteins are directed to locations within or outside of the cell so they can perform their enzymatic or structural functions. In eukaryotes, many proteins, lipids, and carbohydrates are transported through a series of membrane-bound organelles that function in the synthesis, modification, and directed transport of these molecules. This is called the secretory pathway, and is important for key cellular functions including the release of insulin into the blood stream from pancreatic β cells, the production and release of brain chemicals into the synapses of neurons, and the release of immunoglobulins from lymphocytes to name a few.

The secretory pathway begins at the endoplasmic reticulum (ER), where proteins are synthesized and modified. From the ER, proteins are transported to another organelle called the Golgi complex. Unlike the ER, the Golgi is a series of membrane-encased compartments that continue with the modifications required for a protein to function properly. From the Golgi, protein, lipid, and carbohydrate cargo is then sorted and transported to the plasma membrane of the cell or another organelle in the endosome network. In addition to the secretory pathway, a reverse directional transport of cargo also occurs that can recycle materials from the plasma membrane back to the Golgi, consume nutrients and other factors from the extra-cellular environment, or regulate cell surface components. The Golgi complex is formed and maintained by the convergence point of many intracellular

transport pathways; it directs cargo from the ER, the plasma membrane, and endosomes making the Golgi an important transport center.

Although technologies including the electron microscope, fluorescent protein chimeras, and powerful genetic screens have provided great insights into how the secretory and membrane trafficking pathways work, there still remain many unknown aspects of these processes including how transport carriers are formed and how the Golgi is organized.

The Endoplasmic Reticulum

The ER is a web-like network of interconnected membrane tubules and membrane sheets that generate a large, convoluted lumen separated from the cytosol. The ER is where many lipids are synthesized (smooth ER) and where many proteins are synthesized (rough ER). Some mRNA molecules encode an ER signal sequence that when synthesized results in a signal recognition particle (SRP)-dependent translational pause followed by translocation of the mRNA, ribosome, and SRP-bound nascent peptide to the ER membrane where translation is completed (Blobel and Dobberstein 1975; Blobel and Dobberstein 1975; Blobel 1987). The resulting protein is either inserted into the lumen of the ER or into the membrane itself. Proteins are then post-translationally modified by glycosylation and di-sulfide bond formation and, with the help of chaperones, correctly folded into their tertiary structure.

The Golgi Complex

The Golgi complex is just that, complex, and has been surrounded by controversy since its discovery. Camillo Golgi was the first to describe the Golgi as a basket-like structure adjacent to the nucleus in neurons (Golgi

1989). The existence of the Golgi was disputed until the advent of the electron microscope, which allowed the visualization of cellular structures that could not be seen with a light microscope (Dalton and Felix 1954; Dalton and Felix 1956). The function of the Golgi, however, was unclear until George Palade proposed a model for the secretory pathway that included the Golgi complex (Palade 1975). This model was the foundation for our understanding of the Golgi.

The Golgi is a series of stacked membrane-bound compartments called cisternae that each has unique properties. The cisternae are ordered as *cis*-Golgi, *medial*-Golgi, *trans*-Golgi, and TGN. An intermediary compartment called the ER-Golgi-Intermediate Compartment (ERGIC) is a liaison between the ER and the Golgi. Each of the cisternae is functionally and biochemically distinct. From *cis* to *trans* the pH of the lumen decreases and the cholesterol levels in the membrane increase (Anderson, Falck et al. 1984; Bretscher and Munro 1993). The protein content of each cisterna is also specialized for each compartment. Resident enzymes in the *cis* compartment covalently link simple sugar molecules onto proteins. In the *medial* and *trans* compartments, additional complex sugars are added on to this foundation. The Golgi cisternae comprise a series of compartmentalized sequential reactions (Hart 1992).

In most mammalian cells, the Golgi is located adjacent to the nucleus at the microtubule organizing center (MTOC) where the minus ends of microtubules converge. The cytoskeleton is intimately linked to the structure and the function of the Golgi as a nexus for membrane transport. Inhibitors that disrupt the microtubule cytoskeleton network also cause the Golgi to fragment and become decentralized (Thyberg and Moskalewski 1985).

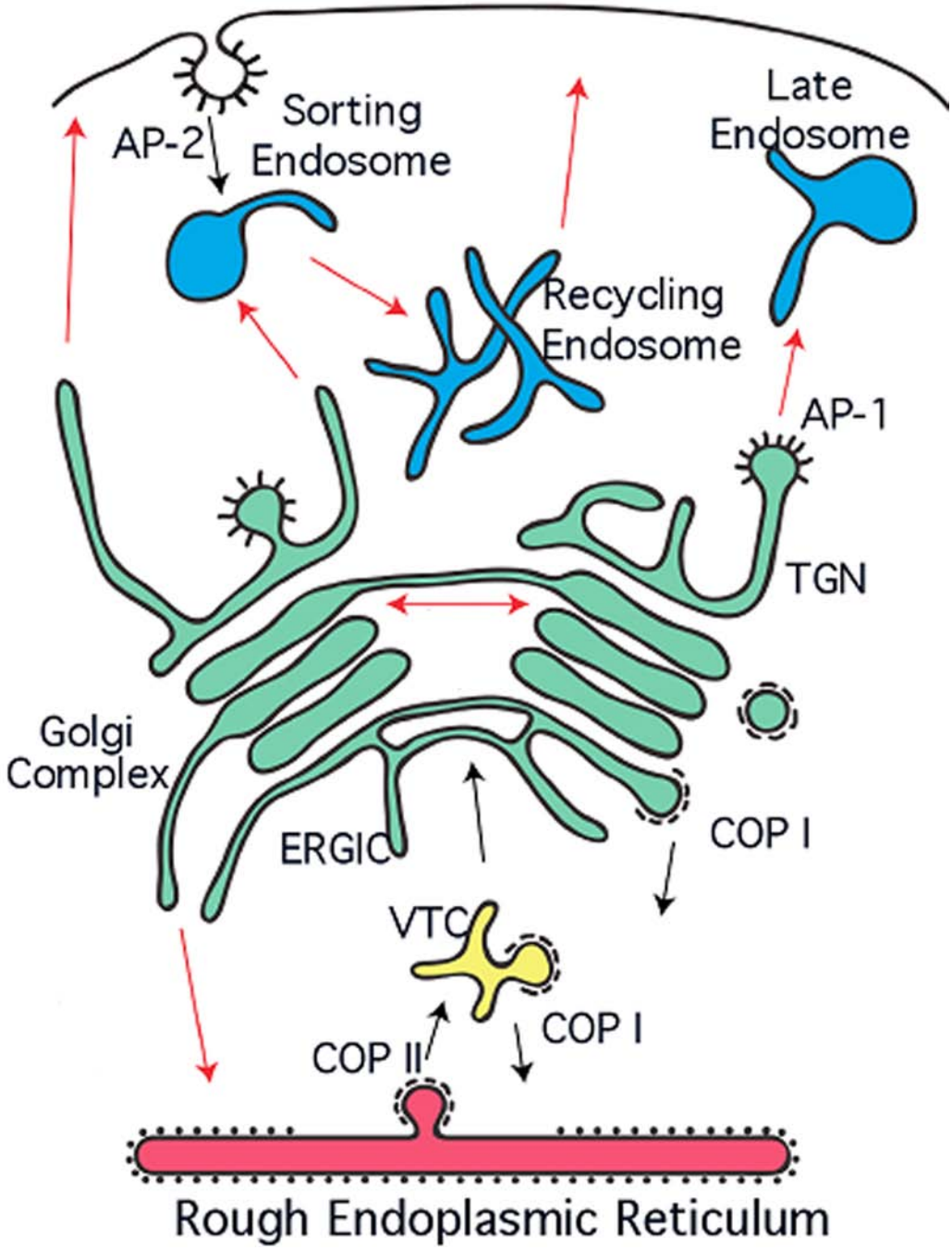
Similarly, when a cell undergoes mitosis and the microtubule network dissociates and scatters, the Golgi scatters too (Kreis 1990). After cell division, the Golgi complex reforms at the MTOC by transport along the reformed cytoskeleton with the aid of dynein, a motor protein that moves from the plus end to the minus end of microtubules (Burkhardt, Echeverri et al. 1997). As expected, disruption of dynein also leads to Golgi fragmentation, which underscores the importance of the cytoskeleton for maintaining Golgi structure (Burkhardt, Echeverri et al. 1997).

In addition to microtubules, Golgi structure is also maintained by a family of proteins containing coiled-coil motifs called golgins. Golgins interact with GTPases, phospholipids, and microtubules to establish Golgi structure, localization, identity and vesicle tethering (Short, Haas et al. 2005).

The Secretory Pathway

The secretory pathway is the directional route through which proteins are synthesized in the ER, processed in the Golgi, and transported to the plasma membrane (Figure 1-1). This process was first proposed by George Palade who followed radioactively labeled proteins through this pathway using biochemistry and EM autoradiography (Palade 1975). Subsequently, yeast genetic manipulation was employed by Randy Scheckman to determine the specific protein factors (“sec” proteins) involved in each major step of the pathway (Novick, Field et al. 1980; Kaiser and Schekman 1990; Deshaies, Sanders et al. 1991). And more recently, Jennifer Lippincott-Schwartz generated protein-GFP chimeras to look at the secretory pathway and its dynamics in real-time (Presley, Cole et al. 1997; Cole, Ellenberg et al. 1998; Presley, Smith et al. 1998). These and other scientific and technological

Figure 1-1: A simplified depiction of the secretory and retrograde pathways. The organelles of the secretory pathway are shown with the ER in red, the ERGIC/VTC in yellow, the Golgi in green, endosomes in red, and the plasma membrane in black. Lipid and protein trafficking between the organelles is accomplished using vesicles including the labeled COPII, COPI, AP-1 clathrin, and AP-2 clathrin. Trafficking pathways using membrane tubules are shown with red arrows.


AP-2
Sorting Endosome
Late Endosome
Recycling Endosome
AP-1
TGN
Golgi Complex
ERGIC
COP I
VTC
COP I
COP II
Rough Endoplasmic Reticulum

advances have shaped our current understanding of the secretory pathway and membrane trafficking.

As mentioned previously, the secretory pathway begins at the ER where proteins are synthesized into the luminal space and correctly folded and modified with the help of chaperone proteins such as BiP, calnexin, and calreticulin. Incorrectly folded proteins are refolded until correct (Bannykh, Rowe et al. 1996). Proteins in the secretory pathway are then concentrated in specialized regions of the ER called ER exit sites (Bannykh, Rowe et al. 1996). ER exit sites contain numerous cup-like membrane outcrops that are surrounded by a highly structured protein coat called COPII coat (Bannykh, Rowe et al. 1996). The COPII proteins form a spherical cage around membranes at ER exit sites encapsulating some of the membrane and the lumen, which is rich in secretory cargo (Mironov, Mironov et al. 2003; Gurkan, Stagg et al. 2006). The result is a spherical vesicle detached from the ER containing various proteins and lipids destined for the secretory pathway (Harter and Reinhard 2000).

The COPII coat proteins disassemble creating naked vesicles that fuse with each other and form a pre-Golgi organelle called the Vesicular-Tubular Compartment (VTC) or the ER-Golgi Intermediate Compartment (ERGIC) (Hauri, Kappeler et al. 2000). How secretory proteins move through the different cisternae of the Golgi is still unclear. However, three hypotheses have been proposed. The predominant hypothesis proposes that the ERGIC compartment grows and matures into a *cis*-Golgi cisterna, which matures into a *medial*-Golgi cisterna, which matures into a *trans*-Golgi cisterna until the cargo is transported to the plasma membrane or other destinations (Pelham 2006). In this maturation model, when a cisterna matures from *cis*-Golgi to

medial-Golgi, *cis*-Golgi proteins will be transported back in COPI coated vesicles and other cisternae behave similarly. Evidence for this model comes from studies that localized resident Golgi processing enzymes, but not secretory cargo, to COPI coated vesicles (Martinez-Menarguez, Prekeris et al. 2001). In addition, live cell imaging reveals that individual Golgi cisternae rapidly convert to more mature elements by acquisition of downstream resident enzymes (Losev, Reinke et al. 2006; Matsuura-Tokita, Takeuchi et al. 2006). In contrast, a second model states that cargo, not resident Golgi proteins, is packaged in COPI coated vesicle and transported through the Golgi in a forward direction (Farquhar and Palade 1998). Evidence for this model comes from studies showing that cargo molecules can be found in COPI vesicles (Orci, Amherdt et al. 2000). Alternatively, a third model suggests that the cisternae are not as distinct as previously thought, but continuously connected via short membrane tubules which act to transport both cargo and resident enzymes using diffusion (Perinetti, Muller et al. 2009). While each model has evidence in support and against, it is clear that both COPI coated vesicle and membrane tubules are important for intra-Golgi transport and Golgi complex integrity.

The TGN is where cargo is sorted for its final destination and where a great deal of regulation occurs. For example, proteins to be transported to lysosomes are modified in the Golgi to contain a mannose-6-phosphate sugars (Dahms, Lobel et al. 1989). Receptors in the TGN recognize that modification and sort those lysosomal enzymes into clathrin coated vesicles that go to endosomes and then lysosomes (Dahms, Lobel et al. 1989). Some proteins and lipids are separated into lipid rafts while others contain specific amino acid sequences that act as zip codes for sorting (Bard and Malhotra

2006). Insulin and neurotransmitters are sorted into organelles called secretory granules that are only secreted from the cell when a cell is signaled to do so (Bard and Malhotra 2006). Insulin release is an example of this regulated secretion. Constitutive secretion is the continuous sorting of cargo into clathrin coated vesicles at the TGN for instant release from the cell at the plasma membrane.

Retrograde Trafficking

Whether the Golgi complex functions using cisternal maturation or vesicle-mediated secretion, a reverse or retrograde pathway must exist to recycle resident ER enzymes and phospholipids. Retrograde pathways also transport proteins and lipids from endosomes to the TGN or from the Golgi to the ER. While much of the retrograde cargo is recycled receptors and enzymes, other proteins and infectious agents also use these routes.

Many proteins that are transported in the retrograde direction from the Golgi to the ER use amino acid sequences that identify where they are to be located. One example is the amino acid sequence KDEL, which is found in many proteins that localize primarily to the ER (Munro and Pelham 1987). If a protein with the KDEL sequence escapes the ER and reaches the Golgi, it is recognized by a KDEL receptor, packaged into COPI coated vesicles, and returned to the ER (Sannerud, Saraste et al. 2003; Stornaiuolo, Lotti et al. 2003). Similarly, a membrane protein with a KKXX sequence will also be transported back to the ER (Sannerud, Saraste et al. 2003; Stornaiuolo, Lotti et al. 2003). For many proteins in the secretory and retrograde pathways, their localization is dynamic. While proteins with a KDEL sequence will mostly be in the ER, some are also present in the Golgi, but at much lower levels.

The ERGIC-53/p58 protein is found in the ER, the ERGIC, and the *cis*-Golgi compartments simultaneously and constantly circulates among all three (Itin, Schindler et al. 1995; Klumperman, Schweizer et al. 1998). ERGIC-53/p58 may also be transported in membrane tubules, not COPI vesicles (Hauri, Kappeler et al. 2000; Sannerud, Saraste et al. 2003). To maintain the location of resident ER and Golgi enzymes, to recycle phospholipids and receptors, and to constitutively transport cargo, the retrograde and anterograde pathways function as two continuous countercurrents. Thus, a challenge in cell biology has been to understand how an organelle can remain stable while its constituent molecules are constantly turning over.

Membrane Vesicles

A great deal of research has focused on how cargo is sorted into transport carriers, how those carriers are formed, and how they fuse with the correct destination. There are three main protein coats for membrane vesicles. The COPII coat contains four structural proteins; Sec13, Sec31, Sec23, and Sec24, which also have roles in binding specific cargo (Barlowe, Orci et al. 1994). COPII vesicles transport cargo from the ER to the Golgi, and much of this cargo contains a specific amino acid sequence or structural motif that allows it to exit the ER (Aridor, Weissman et al. 1998). Sec12 allows the GTPase Sar1 to bind GTP, which signals the assembly of COPII and vesicle budding (Spang 2008).

In the Golgi, the COPI coat consists of α, β, β', γ, ε, and ζ subunits. They assemble when the GTPase Arf1 is bound to GTP (Serafini, Orci et al. 1991). Although COPI vesicles may be involved in either forward transport of secretory cargo or reverse transport of Golgi proteins, COPI components are

critical for maintaining Golgi structure and function. The final group of coated vesicles is clathrin coated vesicles. Clathrin binds to various adaptor proteins on the TGN, endosomes, and plasma membrane (Hirst and Robinson 1998). Different adaptors are involved in different trafficking pathways. Clathrin coated vesicles are similar to COPII and COPI because they also require small GTPases (Bonifacino and Glick 2004).

In addition to coat proteins, vesicles also contain other factors including Rabs, tethers, and SNAREs to identify the contents and destinations of the vesicle. Briefly, these proteins can act as long distance (tethers) and short range (SNAREs) factors that interact with partner proteins on the surface of organelles with which they will promote vesicle-organelle fusion. They aid in matching up the correct cargo-containing vesicles with the correct organelles and they interact to help fuse the vesicle membrane with the target organelle membrane. A vesicle containing a specific v-SNARE will partner with a t-SNARE on the target membrane allowing the vesicle to fuse and release its contents (Sudhof and Rothman 2009).

Membrane Tubules in Intracellular Trafficking

Vesicles are not the only means of transporting cargo between organelles. Membrane tubules are membrane extensions about 50-100 nm in diameter that emanate out from a donor organelle, break off, and deliver cargo to the acceptor organelle, or destination. The role of tubules in organelle structure and function was first examined using fluorescent dyes to look at ER membranes *in vivo*, which revealed an extensive network of tubules that are dependent on microtubules (Terasaki, Song et al. 1984; Terasaki, Chen et al. 1986; Dabora and Sheetz 1988). ER membrane tubules are also dependent

on reticulons (Rapoport 2009). The role of membrane tubules in Golgi structure and function was later seen using a small fungal metabolite brefeldin A (BFA), which rapidly caused tubules to form from Golgi membranes (Lippincott-Schwartz, Yuan et al. 1989; Orci, Tagaya et al. 1991). These studies showed that Golgi membrane tubules induced by BFA are ATP-dependent and fuse with ER membranes. The Rothman lab and others showed that BFA disrupts components of the COPI coat proteins by preventing Arf1 nucleotide exchange (Donaldson, Finazzi et al. 1992), and hypothesized that constitutive trafficking pathways exist between the ER and the Golgi in both anterograde and retrograde directions (Orci, Tagaya et al. 1991).

With advances in technology, these Golgi tubules were visualized in real-time using fluorescently tagged proteins (Sciaky, Presley et al. 1997). Cargo-containing tubules were seen as predominant and ubiquitous features of Golgi membranes that grow and detach in a dynamic way. These tubules are involved in connecting Golgi components and transporting cargo out of the Golgi (Sciaky, Presley et al. 1997). Just like ER tubules, Golgi tubules similarly use both microtubules as a scaffold and motor proteins, but are not completely dependent on them to form (Sciaky, Presley et al. 1997).

In addition to BFA, disruption of proteins involved in trafficking, including Sar1, causes small tubules to form at ER exit sites, and disruption of a TGN vesicle fission component, PKD, results in membrane tubules forming at the TGN (Bielli, Haney et al. 2005; Bard and Malhotra 2006; Brown, Plutner et al. 2008). This indicates roles for tubules in transport and maintenance in the ER, the Golgi, and the TGN, and these tubules may function as a precursor to vesicles.

Membrane tubules are also involved in trafficking pathways between endosomes. Endosomes are involved in the endocytosis of nutrients and other factors from the plasma membrane. Once inside the cell, different factors are sorted and transported to other endosomes, lysosomes, or the TGN. Membrane tubules are important for both endosome transport as well as endosome cargo sorting (Chambers, Judson et al. 2005; Bonifacino and Rojas 2006).

Membrane tubule formation has also been reconstituted *in vitro* (Banta, Polizotto et al. 1995). These studies demonstrated that membrane tubules can form with or without microtubules, but require cytosol and ATP (Cluett, Wood et al. 1993; Banta, Polizotto et al. 1995). From *in vitro* and *in vivo* studies we conclude that membrane tubules are instrumental players in maintaining organelle structure by maintaining a reticular ER or connecting Golgi cisternae, and that tubules are also used to transport cargo between organelles. However, while a great deal is known about the protein coats and regulatory facets of coated vesicles, far less is known about membrane tubules, including factors required for their formation and regulation.

Membrane Curvature

Both membrane vesicles and membrane tubules involved in trafficking require the donor membrane to change shape from a relatively flat surface to a highly curved sphere or tube. There are two general ways in which a membrane can alter its curvature: proteins can bend the membrane extrinsically, and/or the phospholipids themselves can influence membrane shape. In the former case, a protein can alter the shape of a membrane by a few methods. First, a protein with a curved structure can interact with

phospholipids electrostatically and bend the membrane to fit the shape of the protein (McMahon and Gallop 2005). Secondly, the protein can disrupt the phospholipid bilayer by inserting a hydrophobic wedge into the outer leaflet thereby displacing space and generating curved membranes (McMahon and Gallop 2005). Some examples of proteins that can manipulate membrane curvature are BAR-domain proteins, which have arc-shaped structure, and reticulons, which insert loops into the membrane (Shibata, Voss et al. 2008).

In addition to protein factors, the phospholipids that compose a membrane can also influence membrane curvature. Different phospholipids contain different head groups and different acyl chains. Some phospholipid head groups are larger than others making the overall shape of the individual phospholipid less like a cylinder and more like an inverted cone. Likewise, if a phospholipid is missing one of its acyl chains (a lysophospholipid), it too can have a more inverted cone shape. As illustrated in Figure 1-2, altering many phospholipids to inverted cones can generate localized changes in membrane curvature where the membrane bulges outward, or forms positive curvature (Brown, Chambers et al. 2003). Conversely, if a phospholipid has a small head group it can form negative curvature. Lyso-phosphatidylcholine (LPC) is a phospholipid that can generate positive curvature whereas phosphatidic acid (PA) and diacylglycerol (DAG) can generate negative curvature (Brown and Schmidt 2005).

For a vesicle or tubule to form from a donor membrane, positive curvature may be required at the site of bud/tubule formation. Negative curvature may be required at the bud/tubule neck for a vesicle to bud or a tubule to break off from the donor membrane. The bilayer couple hypothesis states that curvature of one membrane leaflet will also curve the opposite

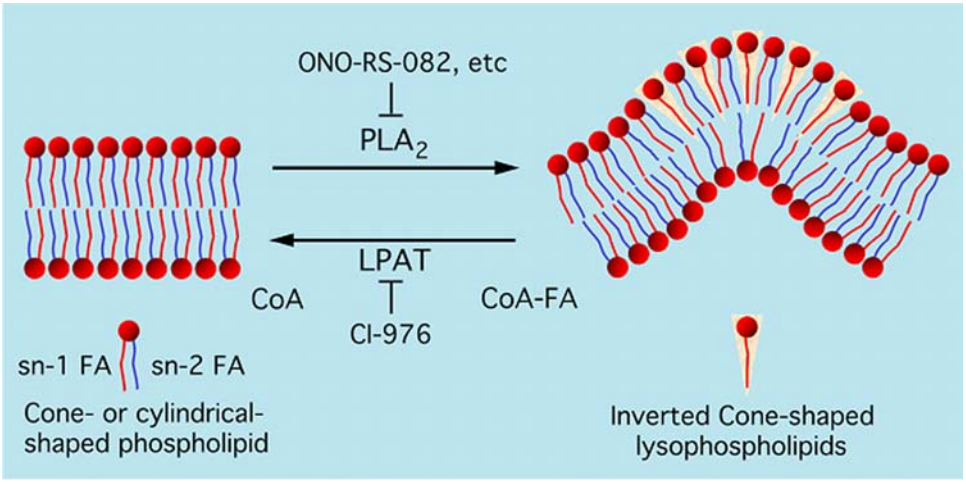
leaflet (Sheetz and Singer 1974). Lipid metabolizing enzymes may play a role in generating the specific phospholipids needed to generate membrane curvature. Without these changes in curvature, the membrane bending energy will be too high for trafficking intermediates to form (Chernomordik and Kozlov 2005). Two phospholipid metabolizing enzymes that are thought to be involved in trafficking are phospholipaseA_2s (PLA_2 enzymes) and lysophospholipid acyltransferases (LPAT) (Brown, Chambers et al. 2003).

Phospholipase A_2 Enzymes

PLA_2 enzymes are a diverse class of enzymes that generate lysophospholipids by hydrolyzing the acyl chain at the sn-2 position of a phospholipid (fig. 1-2). The human genome encodes many PLA_2 enzymes that can be either secreted out of the cell or remain in the cytoplasm (Murakami and Kudo 2002). The functions of these PLA_2 enzymes range from the release of arachidonic acid for signaling pathways to lipid degradation in the lysosome; however, the exact functions of most PLA_2 enzymes are unknown (Balsinde, Winstead et al. 2002; Murakami and Kudo 2002). The role of phospholipases in membrane trafficking became evident when it was discovered that PLA_2 antagonists, including ONO-RS-082 and bromoenol lactone (BEL), can prevent the formation of BFA-stimulated Golgi tubules (de Figueiredo, Drecktrah et al. 1998). While multiple PLA_2 antagonists exhibited this phenotype, those specific for cytosolic calcium-independent PLA_2 enzymes were most effective.

A unique way of stimulating and visualizing Golgi tubules without BFA is to use an *in vitro* system. Isolated Golgi membranes incubated with bovine brain cytosol (BBC) will form tubules (Cluett, Wood et al. 1993; Banta,

Figure 1-2: Membrane curvature can be altered by phospholipid metabolizing enzymes. Phospholipids can alter the relative curvature of membranes. A phospholipid with two acyl or fatty acid (FA) chains has a cylindrical shape while phospholipids with only one FA have an inverted cone shape. Phospholipase A_2 enzymes (PLA_2) can increase inverted cone-shaped phospholipids while lysophospholipid acyltransferases (LPAATs) can generate more cylindrical-shaped phospholipids. Both PLA_2s and LPAATs have known antagonists, which aid in dissecting their physiological functions.


ONO-RS-082, etc
PLA2
LPAT
CoA
CoA-FA
CI-976
sn-1 FA
sn-2 FA
Cone- or cylindrical-shaped phospholipid
Inverted Cone-shaped lysophospholipids

Polizotto et al. 1995). PLA_2 inhibitors, however, are capable of blocking these *in vitro* tubules supporting the idea that PLA_2 enzymes have a critical role in forming Golgi membrane tubules (de Figueiredo, Polizotto et al. 1999).

These PLA_2 antagonists were also capable of blocking Golgi fragmentation when the microtubule depolymerizing agent nocodazole was applied to cells (Drecktrah and Brown 1999). The use of these inhibitors has led to the conclusion that PLA_2 enzymes have a role in re-forming the Golgi complex after mitosis, potentially by generating membrane curvature and membrane tubules to reconnect Golgi mini-stacks (de Figueiredo, Polizotto et al. 1999). PLA_2 inhibitors also prevented the formation of Golgi tubules responsible for constitutive retrograde trafficking to the ER (de Figueiredo, Drecktrah et al. 2000). These results led to the conclusions that Golgi membrane tubules require PLA_2 activity, that Golgi membrane structure is mediated by both microtubules and PLA_2 enzymes, and that Golgi membrane tubules are responsible for maintaining Golgi or ER localization of proteins through constitutive retrograde trafficking pathways (Brown, Chambers et al. 2003). We hypothesize that membrane tubules require PLA_2 activity to generate lysophospholipids, which can form positive membrane curvature (Figure 1-2) – a requirement for tubule growth from the Golgi.

Lysophospholipid Acyltransferases (LPATs)

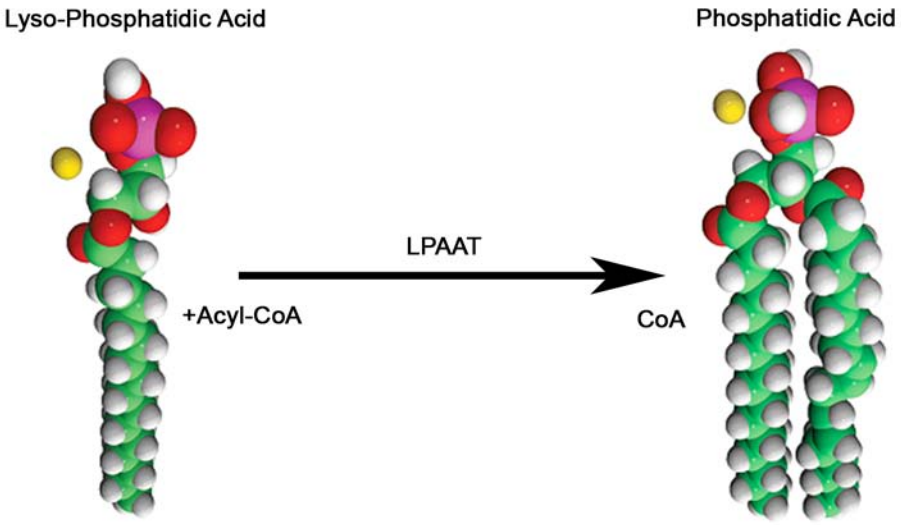
While phospholipase A_2 enzymes appear to be involved in the formation of membrane tubules, a plethora of evidence also indicates a role for LPAT activity in regulating both membrane tubules and vesicles for organelle trafficking. LPATs utilize the lysophospholipid products of a PLA_2 reaction and re-acylate those substrates using acyl-CoA as a fatty acid donor to generate

phospholipids as shown in Figure 1-3. Little is known about specific LPAT enzymes and their activity, but there are nine putative transmembrane LPAT proteins in the human genome that are thought to be lysophosphatidic acid acyltransferases or LPAATs, which is a specific kind of LPAT (Shindou, Hishikawa et al. 2009). Presently they are known to be involved in *de novo* phospholipid and neutral lipid synthesis as well as a role in the Lands' Cycle of phospholipid turnover (Lands 1958; Shindou, Hishikawa et al. 2009).

LPAT activity was suspected to have a role in membrane tubules through the use of a weak ACAT inhibitor, CI-976, which was also found to be a potent LPAT inhibitor (Drecktrah, Chambers et al. 2003; Chambers and Brown 2004). Drecktrah et. al. showed that CI-976 can induce the formation of Golgi membrane tubules *in vivo* and *in vitro* and also enhance retrograde trafficking to the ER (Drecktrah, Chambers et al. 2003). If the cells are pre-treated with a PLA_2 inhibitor, subsequent treatment with CI-976 no longer stimulates tubules (Drecktrah, Chambers et al. 2003). This result supports roles for both PLA_2 and LPATs in regulating tubules.

CI-976 not only induces Golgi tubules, but it was also shown to generate endosome tubules (Chambers, Judson et al. 2005). Additionally, CI-976 inhibits the budding of COPI and COPII coated vesicles *in vitro* (Brown, Plutner et al. 2008; Yang, Gad et al. 2008). LPAT activity is therefore important for multiple steps in the trafficking pathway that involve both tubules and vesicles. The phospholipids generated by LPAT activity and the lysophospholipids consumed by its activity also have an important role in regulating the membrane curvature required for tubule and vesicle biogenesis (Brown and Schmidt 2005).

Figure 1-3: The LPAAT reaction converts a lysophosphatidic acid to phosphatidic acid. A lysophosphatidic acid acyltransferase (LPAAT) will add an acyl-CoA or fatty acid chain to the sn-2 hydroxyl of a lysophosphatidic acid (LPA) to generate a phosphatidic acid (PA). The conversion of LPA to PA is thought to generate net negative curvature in a membrane due to the small size of the PA head group shown in purple. This reaction may involve acid-base catalysis. PA can also be used in the synthesis of other phospholipids and neutral lipids.


Lyso-Phosphatidic Acid
Phosphatidic Acid
LPAAT
+Acyl-CoA
CoA

While the effects of CI-976 indicated a prominent role for LPATs in membrane trafficking, no specific LPAT has been shown to have this activity in the Golgi. The protein endophilin was suspected to be an LPAT and involved in endocytosis at the plasma membrane. Endophilin does indeed have a function in endocytosis, especially at the neuronal synapse (Schmidt, Wolde et al. 1999; Modregger, Schmidt et al. 2003). However, the potential LPAT activity of endophilin is dubious and may not be important for its function (Gallop, Butler et al. 2005). A second protein, C-terminal binding protein3/BFA-ADP ribosylation substrate (CtBP3/BARS), was also thought to be an LPAT with a role in membrane trafficking (Silletta, Di Girolamo et al. 1997; Weigert, Silletta et al. 1999). More recently, however, it is thought that any LPAT activity was due to contamination by other LPATs in an *in vitro* assay (Gallop, Butler et al. 2005). CtBP3/BARS does have an important role in trafficking, but mostly as a vesicle fission-inducing factor. Although discredited, if CtBP3/BARS itself does not have LPAT activity, it may associate with other proteins in the Golgi that do (Bonazzi, Spano et al. 2005; Yang, Lee et al. 2005; Arstikaitis and Gauthier-Campbell 2006).

Transmembrane LPAAT Family of Proteins

Recent scientific advances including cDNA libraries and sequencing of the human genome have resulted in the discovery of many unknown genes including a family that may encode LPAATs. It was first suspected that these LPAATs are involved in phospholipid metabolism due to amino acid sequence similarities to that of a known *E. coli* enzyme with acyltransferase activity (Coleman 1990; Coleman 1992). Although the human proteins are much longer, the region of the protein sequence with similarity to the bacterial

protein is called the acyltransferase or plsC domain. Other notable features of the primary sequence include long stretches of hydrophobic amino acids that may represent transmembrane regions, which are not found in the bacteria protein. LPAAT orthologs can be found in bacteria (plsC), yeast (Slc4), mouse (AGPAT), and other higher eukaryotes.

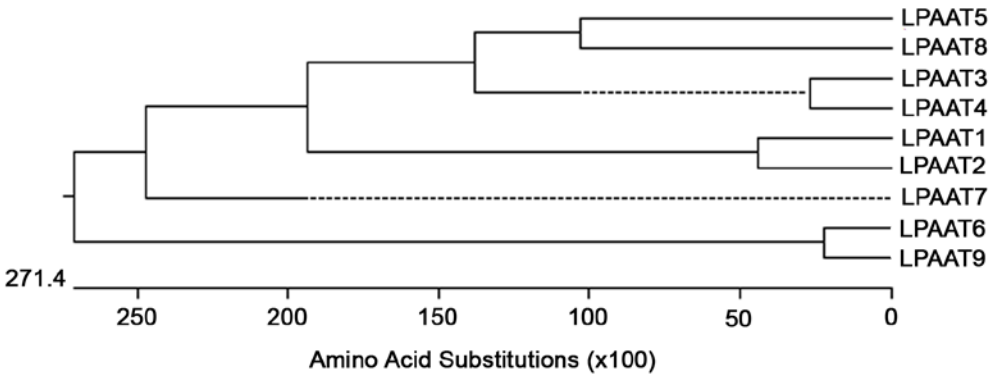
Within the LPAAT acyltransferase domain, there are four motifs that contain amino acids that are highly conserved among different LPAAT family members and among LPAATs of different species (Lewin, Wang et al. 1999). Two of these motifs are quite small while two of them are larger and contain acidic and basic residues. These conserved residues may be important since the acyltransferase reaction may require an acidic motif to stabilize the hydrogen and a basic residue to stabilize the oxygen atoms that make up the sn-2 hydroxyl of the lysophospholipid that is being acylated (Leung 2001). Although this has not been empirically confirmed, it is hypothesized that an LPAAT would utilize an acid-base catalysis mechanism. The first conserved motif has an amino acid sequence NHQSxxD, which contains both acidic (aspartate) and basic (histidine) residues. The second conserved motif EGTRxxG also contains acidic (glutamate) and basic (arginine) residues. Therefore, either one of these motifs, or both, or neither could be relevant for catalytic activity in these LPAATs. Some initial studies have indicated that these regions are important for LPAAT function in the bacterial protein (Morand, Patil et al. 1998). Alternatively, conserved regions may also be important for substrate binding, especially if the substrates are charged phospholipids (Yamashita, Nakanishi et al. 2007).

Currently, nine potential human transmembrane LPAAT proteins have been identified. Most of these nine are known only by their DNA sequences,

but a few have been studied. Since little is known about these LPAATs, a clear nomenclature scheme has not yet been established. Originally the LPAATs were called AGPATs (1-acyl *sn*-glycerol-3-phosphate *sn*-2-acyltransferases) and given greek letters based on the order in which they were discovered. Since then, numbers have replaced the greek letters and, in some cases, more specific abbreviations have replaced the 'LPAAT/AGPAT' name as individual functions become clear. For example, AGPAT alpha has been shown to be a lysophosphatidic acid acyltransferase, so it has been named LPAAT1. For the sake of simplicity, in this thesis the individual family members will be referred to as LPAAT1-9 (Shindou and Shimizu 2009). Figure 1-4 shows the phylogenetic relationship between the members of the LPAAT family.

The first LPAAT to be cloned and studied was LPAAT1. LPAAT1 was shown to be a lysophosphatidic acid acyltransferase that is expressed in all human tissues including the liver and pancreas, where expression is particularly high (Eberhardt, Gray et al. 1997). It has also been suggested that LPAAT1 may catalyze a reverse reaction under conditions of excess phosphatidic acid (Yamashita, Kawagishi et al. 2001). LPAAT3 also appears to be expressed in all tissues; however, LPAAT2, LPAAT4, LPAAT5, LPAAT8 and LPAAT9 are expressed only in some tissues indicating that these LPAATs may have tissue-specific functions (Li, Yu et al. 2003; Lu, Jiang et al. 2005; Agarwal, Barnes et al. 2006; Agarwal, Sukumaran et al. 2007; Chen, Kuo et al. 2008). LPAAT2 has also been studied and found to be involved in congenital lipodystrophy, a condition that results in the loss of adipose tissue (Haque, Garg et al. 2005). LPAAT6 may have a role in synthesizing phospholipids and neutral lipids in skin and mammary tissue (Beigneux, Vergnes et al. 2006;

Figure 1-4: Phylogeny of the nine members of the transmembrane LPAAT family. Amino acid sequences for LPAAT1-9 were compared using Clustaw V to generate a branched diagram representing the sequence divergence. While some LPAATs have some sequence similarity, in general there is considerable variation between the nine proteins in this family of putative LPAATs.


LPAAT5
LPAAT8
LPAAT3
LPAAT4
LPAAT1
LPAAT2
LPAAT7
LPAAT6
LPAAT9
271.4
250
200
150
100
50
0
Amino Acid Substitutions (x100)

Vergnes, Beigneux et al. 2006). Except for the two conserved motifs, there is very little sequence similarity. This implies that some LPAATs may be involved in the *de novo* biosynthesis of phospholipids and neutral lipids, and that although multiple LPAATs are expressed in human tissues, they may have individual non-complementary functions.

Overall Goals

Previous work has indicated that LPAAT activity is required for the formation of membrane vesicles and membrane tubules for intracellular trafficking pathways. Prior to the recent sequencing of LPAAT7, LPAAT8, and LPAAT9, the only known LPAATs were LPAAT1-6. Since LPAAT1 and LPAAT2 had already been cloned and localized to ER membranes, I focused my efforts to identify and characterize a transmembrane LPAAT from among the other members that may be involved in membrane trafficking. As will be discussed in Chapters 2-4, I have found that LPAAT3 is a novel transmembrane LPAAT that is involved in Golgi trafficking and phospholipid metabolism. I examined LPAAT3 using biochemical, molecular, and cell biological methods to investigate the regulation of Golgi structure and function. Additionally, the role of PLA_2 activity in regulating the dynamics of membrane tubules in the TGN as well as trafficking from the TGN will be addressed in Chapter 5. Through these two series of experiments, I show that phospholipid metabolism and turnover in organelle membranes is a critical feature of organelle structure, trafficking, and dynamics.

CHAPTER 2

LPAAT3, a Member of the LPAAT Family of Enzymes, is a Golgi-Localized Lysophosphatidic Acid Acyltransferase with Two Transmembrane Domains

Introduction

Through the use of inhibitors such as CI-976, but no specific protein has been identified to perform these functions. LPAT activity has been linked to *de novo* phospholipid metabolism and the formation of both membrane tubules and vesicles. The fission-promoting CtBP/BARS and endophilin were suspected to have LPAT activity (Bonazzi, Spano et al. 2005), but those claims have since been disputed (Gallop, Butler et al. 2005).

Previous studies from our lab suggested the presence of a CI-976-sensitive LPAT that is very tightly associated with Golgi membranes (Chambers and Brown 2004). One protein in a family of transmembrane LPAATs, LPAAT3, appears to co-localize with Golgi complex proteins. In this chapter, the biochemical properties of LPAAT3 are explored including its activity and topological orientation in membranes. LPAAT3 was tested for LPAT activity, substrate specificity, and specific amino acid residues required for activity. Biochemical and fluorescent approaches were used to map transmembrane regions in the primary sequence.

Materials and Methods

Cell Culture and Immunofluorescence

All cells were grown in MEM + 10% NuSerum (or FBS) in a 37°C chamber with 5% CO_2. Cells grown on coverslips were transfected with

pEGFP N-1 LPAAT3 and c-myc or HA epitope tags inserted into the sequence. Cells were then fixed in 3.7% formalin in PBS, washed in PBS and permeabilized with either 0.1% Triton x-100 in PBS or a digitonin solution (3 μg/ml digitonin, 0.3 *M* sucrose, 5 m*M* $MgCl_2$, 120 m*M* KCl, 0.14 m*M* $CaCl_2$, 2 m*M* EGTA, 25 m*M* HEPES pH to 7.6 with KOH). Cells were then incubated with diluted primary antibodies: 9B11 anti c-myc (1:1000), ABR anti PDI (1:1000), or anti HA (1:100) followed by secondary antibody anti mouse or anti rabbit TRITC (1:100). Peptide antibody for LPAAT3 was designed and generated by Pacific Immunology (Ramona, CA). The antibody was designed against amino acids 254-270: CVRRFPLEDIPLDEKEA. Cells were then viewed and imaged using the Zeiss Axioscope 2.

Cloning/Mutagenesis

LPAAT3 (AGPAT3) cDNA was obtained in pCMV-SPORT6 from the EST collection of the IMAGE Human library (Invitrogen, Carlsbad, California). Using PCR and the multiple cloning site of pEGFP N-1 (Clontech, Mountain View, CA), LPAAT3 was inserted in frame with the EGFP C-terminus before the stop codon. To make the catalytic mutant of LPAAT3 and to insert either c-myc or HA sequences within LPAAT3, Quick Change II (Stratagene) mutagenesis kit with mutagenic primers was used. All constructs were verified by double stranded DNA sequencing. DNA constructs were transfected into cells grown on coverslips using Fugene 6 transfection reagent (Roche, Nutley, NJ) and processed for immunofluorescence experiments within 24-48 h unless otherwise indicated.

LPAAT Assay

Cells were scraped from four 500 cm^2 dishes at 4°C and resuspended in with ST homogenization buffer (0.25 *M* sucrose, 1 m*M* EDTA, and 10 m*M* Tris-Cl pH 7.4) solution such that the cell pellet was 20% of the total volume. Cells were lysed using a Balch-Rothman homogenizer with 22 μm clearance. Nuclei were removed by centrifugation at 2,000 rpm for 5 min in a clinical tabletop centrifuge. Homogenized post-nuclear supernatant (PNS) aliquots were stored at -80°C.

To further isolate Golgi membranes, 62% w/v sucrose solution was added to the PNS to a final sucrose concentration of 37% w/v. Golgi membranes were enriched by flotation by overlaying the PNS with 35% sucrose (7 mL) and 29% sucrose (4 mL) layers (10 m*M* Tris, 1 m*M* EDTA) and centrifuged using an SW28.1 rotor in a Beckman Coulter Optima LE-80K ultracentrifuge (Beckman Coulter, Fullerton, CA) at 25,000 rpm for 2.5 h at 4°C with no brake. Golgi membranes were harvested from the 35%-29% sucrose interface and stored at -80°C.

LPAAT assays were performed as previously described using PNS or isolated Golgi membranes (Drecktrah, Chambers et al. 2003; Chambers and Brown 2004). Lysophospholipids (12 nmol/reaction) and ^{14}C-palmitoyl-CoA (0.04 μCi/reaction) were prepared by suspending them in assay buffer (150 m*M* NaCl, 1 m*M* EDTA, and 10 m*M* Tris, pH 7.4) and sonicating in a bath sonicator for 7 min in the dark. PNS or Golgi (500 μg of protein) were diluted in 125 μl of assay buffer and warmed to 37°C. The phospholipid mixture was then added to the PNS or Golgi (total volume of 200 μl) and incubated at 37°C for 1h.

To stop the reaction and extract the phospholipids, 1 mL of chloroform/methanol/water (316/632/53) was added to the reaction mixture so the final ratio of chloroform/methanol/water was 1/2/0.8 by volume. Each sample was vortexed for 5 min followed by the addition of 300 μl of chloroform and 300 μl of water to each sample. Each sample was vortexed again for 5 min. After 5 min, samples were vortexed again followed by centrifugation at 2,000 rpm in a clinical centrifuge for 5 min. The lower phase (approx. 600 μL) was removed and placed in a new tube. The phospholipids in the lower phase were then dried using argon until the solution was evaporated.

To separate samples, thin layer chromatography (TLC) plates were washed and dried with a chloroform/methanol/acetate/water solution (60/50/1/4). The dried samples were resuspended in 10 μL of chloroform/methanol (1:1) and spotted onto the TLC plates. Samples were resolved with the same solution used to wash the plates. When the resolution buffer reached the top of the TLC plate, the plate was removed and allowed to dry. To look at all phospholipids, plates were stained with a copper (II) sulfate solution (3% copper sulfate, 8% phosphoric acid, water) or exposed to a phosphor screen to detect radio-labeled lipids. All phospholipids were from Avanti Polar Lipids (Alabaster, AL), chromatography reagents were from Alltech Chromatography (Deerfield, IL) and radiolabeled palmitoyl-CoA [palmitoyl-1-^{14}C] was from PerkinElmer Life Sciences (Boston, MA). Sample tubes were boro-silicate glass tubes with Teflon caps. PNS and/or Golgi membranes were measured for protein concentrations using a Bradford assay.

Protease Protection Assay

Cells were grown on 300 cm^2 plates, scarped to harvest, and spun down in microcentrifuge tubes. The pellet was resuspended in 240 µL PBS and homogenized by passage though a 26 2/3 gauge syringe. Nuclei were pelleted from the homogenate by microcentrifugation at 600 X g. The supernatant was collected and microsomes, Golgi and other membranes were further pelleted by centrifugation at 100,000 x g for 30 min. The pellet was resuspended in 240 µL PBS and to this was added 2 µg/ml trypsin and 0.1% Triton X-100 when appropriate (Stone, Levin et al. 2006).

Statistics

Error bars on graph represent standard deviation values for a minimum of three hundred cells counted and a minimum of three independent experiments.

Results

LPAAT3 Partially Localizes to Golgi Membranes

To identify a transmembrane LPAAT that would be a candidate for regulating Golgi structure and function, LPAAT3, LPAAT4, LPAAT5, and LPAAT6 were cloned into GFP expression vectors. Each construct contained a GFP fused to the C-terminus of the protein. As shown in Figure 2-1, LPAAT3 appeared to co-localized with an antibody marker for the Golgi complex, LPAAT5 co-localized with a marker for mitochondria, and LPAAT4 and LPAAT6 co-localized with antibody markers for the ER. This initial screen indicated that LPAAT3 may be functioning at the Golgi membranes. To verify these results, a more comprehensive analysis was done. First, an antibody

Figure 2-1: The localization of LPAAT3, 4, 5, and 6-GFP to organelle markers. LPAAT3, 4, 5, and 6 were fused with green fluorescent protein (GFP) and compared to markers for different organelles. LPAAT3-GFP localized with Golgi markers, LPAAT5-GFP localized with mitochondria markers, and LPAAT4 and 6 localized with ER markers.

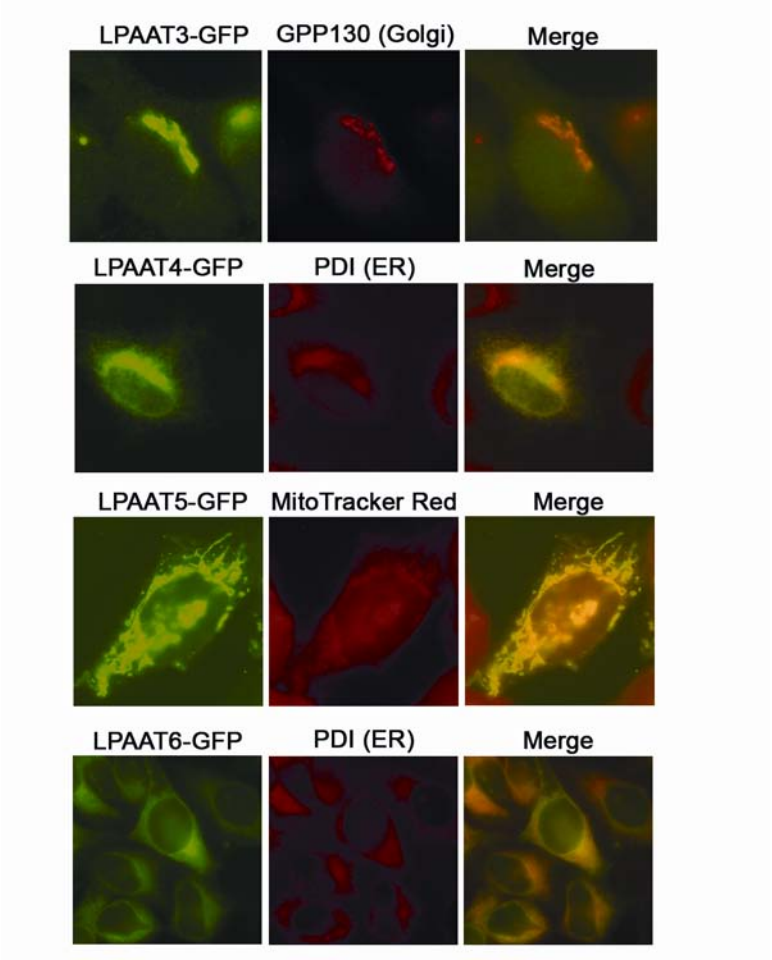
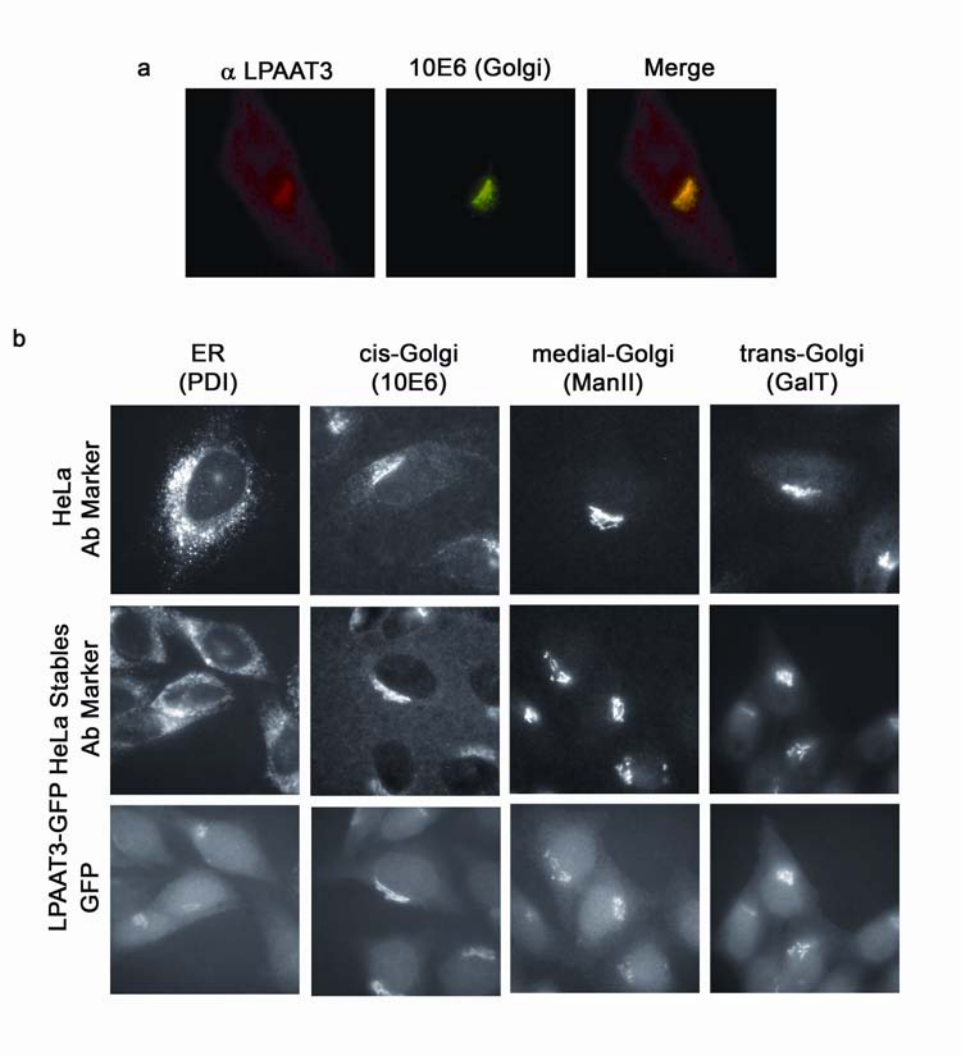

LPAAT3-GFP
GPP130 (Golgi)
Merge
LPAAT4-GFP
PDI (ER)
Merge
LPAAT5-GFP
MitoTracker Red
Merge
LPAAT6-GFP
PDI (ER)
Merge

Figure 2-2: LPAAT3 Localization. (a) An antibody raised against LPAAT3 co-localized with the *cis*-Golgi marker 10E6. (b) HeLa cells were generated to stably express LPAAT3-GFP. HeLa cells and HeLa cells expressing LPAAT3-GFP were incubated with antibodies to the ER marker Protein Disulfide Isomerase (PDI), the *cis*-Golgi protein 10E6, the *medial*-Golgi protein Mannosidase II (ManII), and the *trans*-Golgi protein Galactosyltransferase (GalT). LPAAT3 co-localized with all of the Golgi markers.


a
α LPAAT3
10E6 (Golgi)
Merge
b
ER
(PDI)
cis-Golgi
(10E6)
medial-Golgi
(ManII)
trans-Golgi
(GalT)
HeLa
Ab Marker
LPAAT3-GFP HeLa Stables
Ab Marker
GFP

generated against human LPAAT3 was incubated with cells to locate endogenous LPAAT3. In Figure 2-2a, the LPAAT3 antibody co-localized with the *cis*-Golgi marker 10E6. Both HeLa cells and Clone 9 cells (rat hepatocytes) were transfected with LPAAT3-GFP and selected for individual cells stably expressing the recombinant protein. In both cell lines, LPAAT3 was located in the Golgi membranes. Using those LPAAT3-GFP HeLa cells, different markers for the *cis*-Golgi, the *medial*-Golgi, and the *trans*-Golgi were compared to LPAAT3-GFP. Those markers all localized with LPAAT3 (Figure 2-2b).

LPAAT3 Primary Sequence and Domains

LPAAT3 therefore may be a source of LPAAT activity which has been shown to be important for Golgi trafficking. Human LPAAT3 is expressed in multiple tissues including the liver, brain, heart, lung and spleen (Lu, Jiang et al. 2005). More recently, LPAAT3 has been implicated in multiple activities within testicular cells including roles in phosphatidic acid and phosphatidylinositol metabolism (Yuki, Shindou et al. 2009). To further examine LPAAT3 and the transmembrane LPAAT family, *in silico* methods were used to conduct primary sequence analysis. Figure 2-2 shows the relationship between LPAAT3 among higher eukaryotes including the previously mentioned conserved motifs (blue boxes) and the putative transmembrane regions (black arrows). While the LPAAT family is quite diverse (Fig. 1-4), LPAAT3 itself is conserved among higher eukaryotes. The simplified domain structure of LPAAT3 is shown in Figure 2-4 including potential transmembrane domains and the acyltransferase domain. Two

Figure 2-3 LPAAT3 Sequence Alignment. The primary amino acid sequences for multiple eukaryotes were aligned using Clustaw V. LPAAT3 is highly conserved between species. Black arrows indicate potential transmembrane regions. Horizontal blue boxes indicate two conserved consensus motifs found in all LPAAT3 sequences, all LPAAT family members, *E. coli* PlsC, and *S. cerevisiae* SLC4 orthologs.

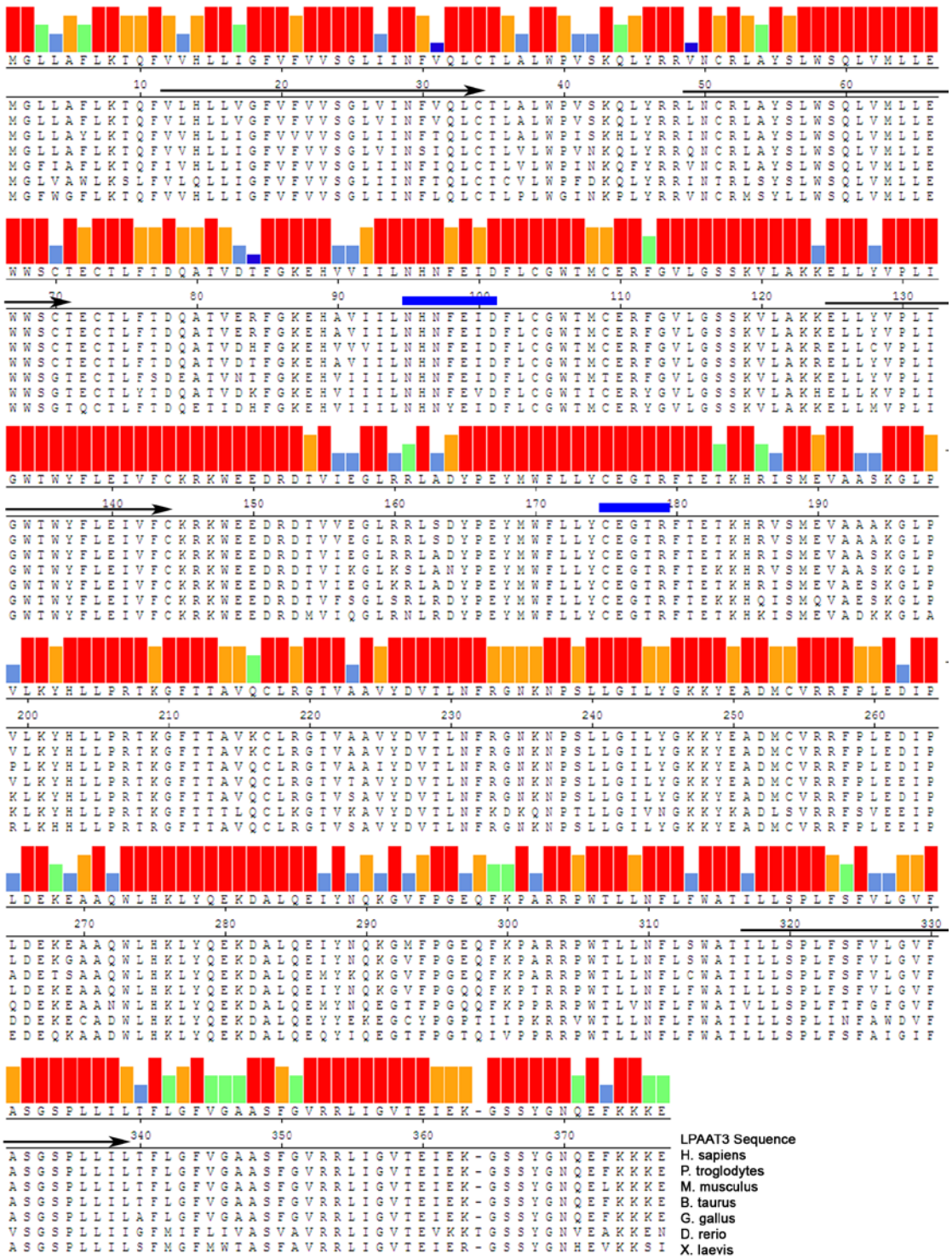
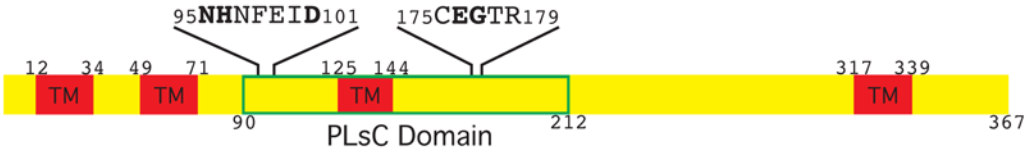

LPAAT3 Sequence
H. sapiens
P. troglodytes
M. musculus
B. taurus
G. gallus
D. rerio
X. laevis

Figure 2-4: LPAAT domains. Like other LPAATs, LPAAT3 is predicted to contain transmembrane (TM) domains and an acyltransferase or PLsC domain. Within the PLsC domain, two conserved motifs are shown that contain acidic and basic amino acid residues. Amino acids mutated to alanines are shown in bold.


95NHNFEID101
175CEGTR179
12
34
49
71
125
144
317
339
TM
TM
TM
TM
90
212
367
PLsC Domain

highly conserved amino acid motifs within the acyltransferase domain are shown in bold type.

LPAAT3 is a Lysophosphatidic Acid Acyltransferase (LPAAT)

To determine if LPAAT3 is indeed an acyltransferase and to determine its substrate specificity, PNS from cells overexpressing LPAAT3 were incubated with various lysophospholipids and a ^{14}C-palmitoyl-CoA fatty acid. LPAAT3 is expected to transfer the radiolabeled palmitoyl-CoA to a lysophospholipid acceptor to generate a phospholipid. The products can then be resolved using thin layer chromatography to separate and detect radioactive phospholipid products.

After quantification, a two-fold increase in the amount of radioactive phosphatidic acid (PA) was seen in cells overexpressing LPAAT3, but not in mock transfected cells (Figure 2-5). Radiolabeled phosphatidylcholine (PC) was the most abundant, as expected, but its levels did not change in cells overexpressing LPAAT3. Other phospholipids including lysophosphatidylserine (LPS), lysophosphatidylethanolamine (LPE), and lysophosphotidylcholine (LPC) showed no significant change between transfected and untransfected cells (Figure 2-5). Cells expressing LPAAT6, a known LPAAT, were also assayed as a positive control for LPAAT activity. From these data, it appears that LPAAT3 is in fact an LPAAT and uses lysophosphatidic acid as a substrate for its acyltransferase activity.

Figure 2-5: Measuring the acyltransferase activity of LPAAT3. PNS from control cells and cells overexpressing either LPAAT3 or LPAAT6 were incubated with C^{14} radio-labeled palmitoyl-CoA and lyso-phospholipid substrates *in vitro*. Both LPAAT3 and LPAAT6 appeared to have lysophosphatidic acid acyltransferase activity (LPA-AT). There were no changes between control PNS and transfected lysates for lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), or lysophosphatidylserine (LPS) acyltransferase activity.

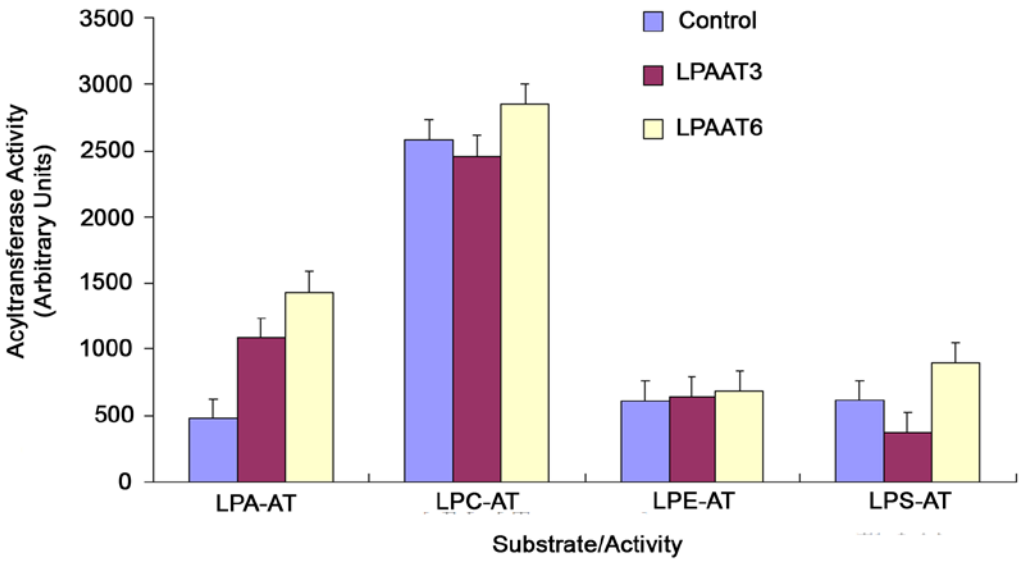
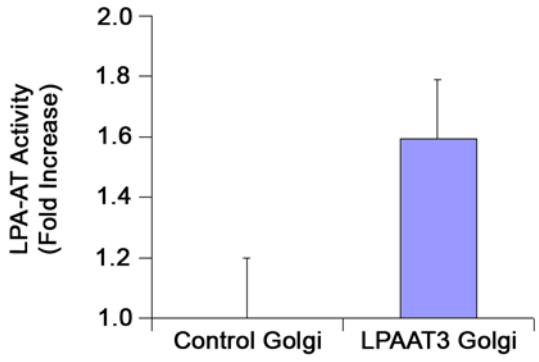

Control
LPAAT3
LPAAT6
Acyltransferase Activity
(Arbitrary Units)
3500
3000
2500
2000
1500
1000
500
0
LPA-AT
LPC-AT
LPE-AT
LPS-AT
Substrate/Activity

Figure 2-6: LPAAT activity in Golgi from cells overexpressing LPAAT3. Golgi membrane from control cells and cell overexpressing LPAAT3 were isolated by sucrose gradient high-speed centrifugation. Golgi from cells with LPAAT3 have elevated LPA-AT activity when compared to control.


LPA-AT Activity
(Fold Increase)
2.0
1.8
1.6
1.4
1.2
1.0
Control Golgi
LPAAT3 Golgi

Cells Overexpressing LPAAT3 have elevated LPAAT activity in Golgi Membranes

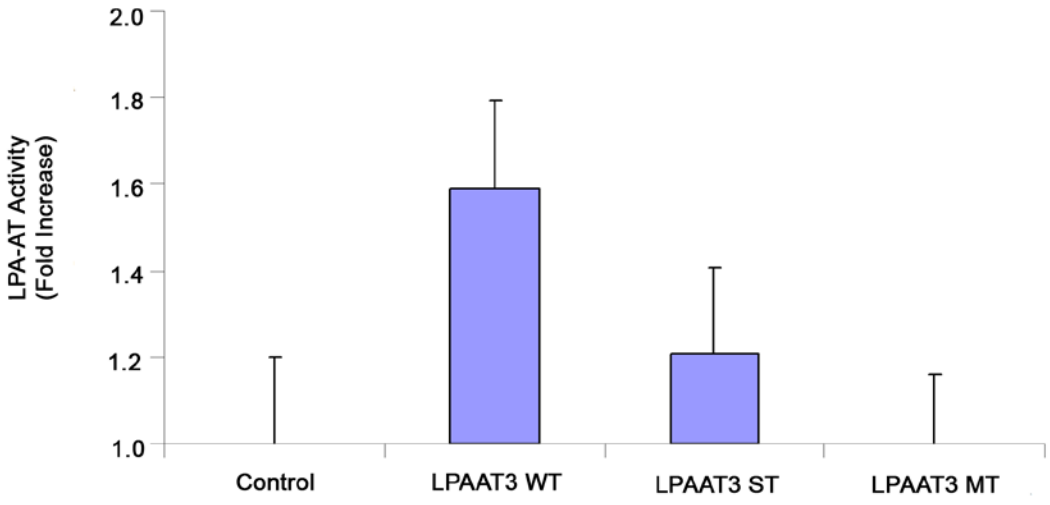
Since LPAAT3 partially localizes to Golgi membranes, changes in LPAAT activity was tested in Golgi membranes from cells transfected with LPAAT3. For this purpose, Golgi membranes were further separated from PNS by sucrose gradient centrifugation. Additionally, only endogenous lysophospholipids were made available as potential acceptors of the ^{14}C-palmitoyl CoA. Golgi membranes from cells overexpressing LPAAT3 had higher levels of LPAAT activity compared to untransfected cells (Figure 2-6). These data indicate that not only is LPAAT3 partially located in Golgi membranes, but it can also alter the phospholipid makeup of the membrane by increasing the amounts of phosphatidic acid and decreasing the amounts of lysophosphatidic acid.

Mutagenesis of Two Conserved Motifs can abolish LPAAT activity in LPAAT3

Within the acyltransferase domain of LPAAT3 there are two motifs common to all LPAAT family members (Figure 2-4). These motifs are suspected to be important for catalysis and substrate binding. The acyltransferase reaction may be an acid-base catalysis. Using site-directed mutagenesis, critical conserved amino acids including acidic and basic residues were changed to alanines. Within the first motif, NHNFEID was changed to NAAFEIA and within the second motif, CEGTR was changed to CAATR. This mutant form of LPAAT3 was tested for LPAAT activity.

In Figure 2-7, Golgi membranes from cells transiently overexpressing either LPAAT3 wild type (WT) or LPAAT3 mutant (MT) are compared to mock

Figure 2-7: Identification of amino acid motifs in LPAAT3 critical for LPAAT activity. Acidic and basic residues in the acyltransferase domain of LPAAT3 were mutated. Golgi membranes from cells transiently transfected with this mutant version were compared to Golgi membranes from cells with LPAAT3 wild type (WT) and from control cells. Unlike the wild type, the LPAAT3 mutant (MT) did not have elevated LPA-AT activity. Cells stably expressing LPAAT3 (ST) also were shown to have an increase in LPA-AT activity, although less than transiently transfected cells. Activity has been normalized to protein concentration and expression levels of LPAAT3 WT and LPAAT3 MT.


LPA-AT Activity
(Fold Increase)
2.0
1.8
1.6
1.4
1.2
1.0
Control
LPAAT3 WT
LPAAT3 ST
LPAAT3 MT

transfected cells for their LPAAT activity using endogenous lysophospholipid acceptors. While there was a greater than 1.5 fold increase in LPAAT activity for LPAAT3 WT, there was little change from mock transfected cells for the LPAAT3 MT Golgi. The Golgi membranes from cells stably expressing LPAAT3 (ST) were also assayed for LPAAT activity, and indeed showed an increase in acyltransferase activity, albeit less robust than transiently transfected cells. These data show that at least one, and possibly two, conserved motifs within the acytransferase domain are important for catalytic activity and that cells with stably expressed LPAAT3 are viable. Stably expressing cells and a catalytically dead version of LPAAT3 will be useful in dissecting the physiological functions of LPAAT3.

Identification of Transmembrane Regions Using Selective Membrane Permeability and Immunofluorescence

Human LPAAT3 and other LPAAT family members are all thought to be transmembrane proteins; however, each LPAAT is predicted to have varying numbers of transmembrane regions and varying locations for those regions. The location of transmembrane regions has implications for positioning the acyltransferase domain in the luminal or on the cytoplasmic side of the membrane bilayer and may therefore have an effect on the physiological roles of LPAAT3. Analysis of the primary sequence of LPAAT3 by various computer algorithms predicts a variable number and location of putative transmembrane domains. These predictions do not allow us to make any definitive conclusions about the topology of LPAAT3; however the potential locations of these transmembrane regions are depicted in Figure 2-4. To empirically determine which sequences of LPAAT3 are exposed to the cytoplasm and

which are inside the lumen, a method developed by Jennifer Lippincott-Schwartz using antibody recognition of epitopes was used (Lorenz, Hailey et al. 2006).

Specific amino acid sequences that are easily recognized by common antibodies were inserted into the wild type amino acid sequence of LPAAT3. The sequences used include c-myc and HA epitopes and GFP. The first two are short and unlikely to disrupt membrane insertion or folding and the last is known to leave LPAAT3 functional if fused to the C-terminus. Cells transfected with LPAAT3 DNA containing each of these sequences were selectively permeabilized in either a digitonin solution or a Triton X-100 solution. At the detergent concentrations used, digitonin permeabilizes only the plasma membrane, thus allowing antibodies to enter the cell and bind to sequences exposed to the cytoplasm. Triton X-100 solution permeabilizes all membranes and thus exposes all sequences to antibodies whether inside the lumen or in the cytoplasm.

In Figure 2-8, it appears as though the regions around myc11, myc41, HA93, and GFP367 are all exposed to the cytoplasm since they were identified by antibody binding and secondary fluorescent antibody when incubated with digitonin (see Fig. 4-10 for tag locations). The regions around myc175, however, were not exposed in the cytoplasm and therefore were in the lumen. These results predict two possible transmembrane passes in the sequence of LPAAT3. Protein disulfide isomease (PDI) is a resident ER protein in the lumen, which was used as a control.

Figure 2-8: The use of immunofluorescence to determine the membrane topology of LPAAT3. Epitope tags were inserted into the LPAAT3 amino acid sequences at specific locations indicated by the tag and number. These were individually transfected into HeLa cells and the cells were permeabilized with either digitonin or Triton X-100 and treated with corresponding antibodies. Cells that showed fluorescence with digitonin processing are inferred to contain that epitope tag in the cytoplasm. If cells are not fluorescent with digitonin, the tag is inferred to reside in the lumen.

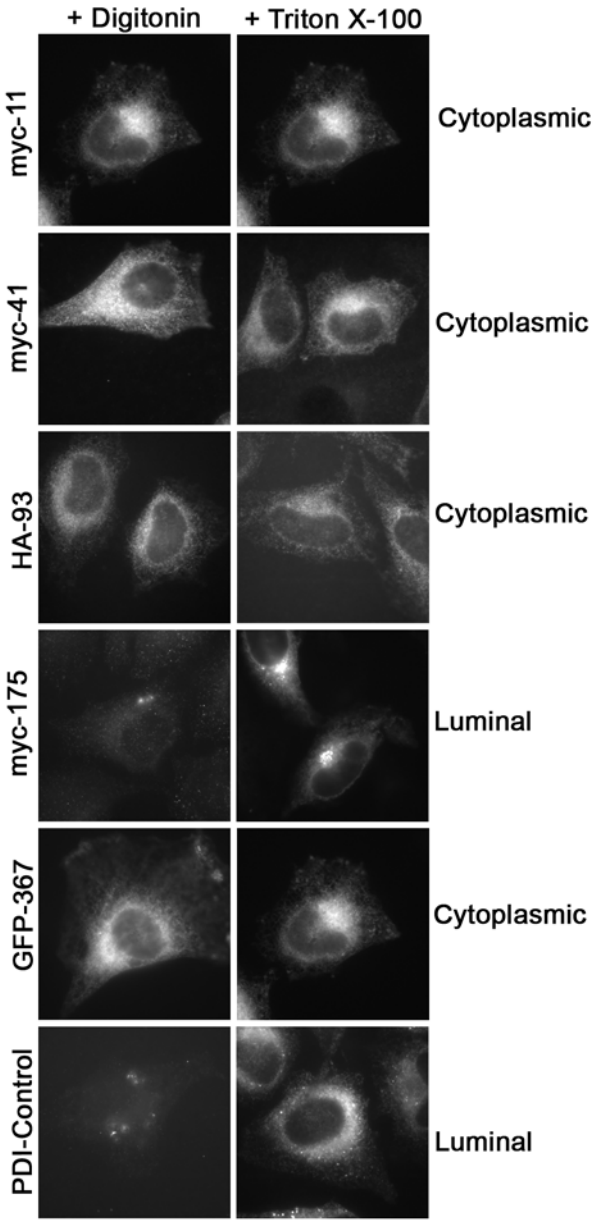
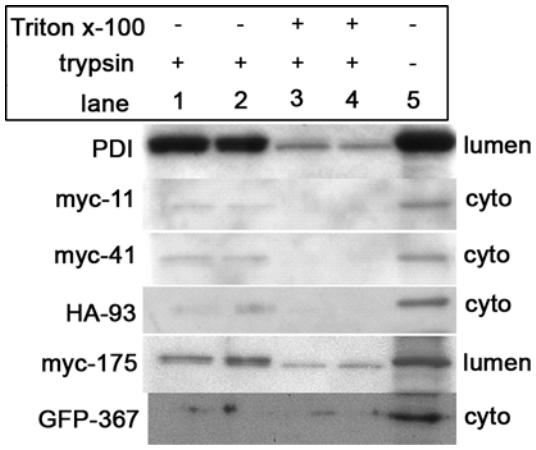

+ Digitonin
+ Triton X-100
myc-11
Cytoplasmic
myc-41
Cytoplasmic
HA-93
Cytoplasmic
myc-175
Luminal
GFP-367
Cytoplasmic
PDI-Control
Luminal

Figure 2-9: Protease protection assay to determine LPAAT3 topology. Organelles from cells transfected with LPAAT3 genes encoding epitope tags were isolated and subjected to trypsin proteolysis. Epitope were detected by western blot. In samples with Triton X-100 all tags are expected to be digested, in samples with digitonin only cytosolic tags are expected to be digested, and in samples with no trypsin tags are expected to be preserved. Lanes 1 and 2 are duplicates, and lanes 3 and 4 are also duplicates.


Triton x-100 - - + + -
trypsin + + + + -
lane 1 2 3 4 5
PDI
lumen
myc-11
cyto
myc-41
cyto
HA-93
cyto
myc-175
lumen
GFP-367
cyto

Identification of Transmembrane Regions by Protease Protection Assay

The protease protection assay is the same fundamental idea as above, except that instead of testing for exposed regions using antibodies *in vivo*, exposed areas were subjected to proteolysis *in vitro*. I used two methods to determine the topology of LPAAT3 to give confidence in the final model. In-tact Golgi and microsomes from cells transfected with each of the constructs described above were incubated with trypsin or trypsin and Triton X-100. If the epitope is not detected by western blot analysis, then it is presumed to be exposed to the cytoplasm and digested. If a strong band is detected, then the membrane is protecting that sequence from proteolysis. The results indicate that myc11, myc41, HA93, and GFP367 are all digested by trypsin and myc175 is not (Fig. 2-9). These data are consistent with the fluorescence data and give us a model of LPAAT3 including the number of transmembrane regions and, along with hydrophobic domain information, the approximate locations of these regions (Fig. 2-10).

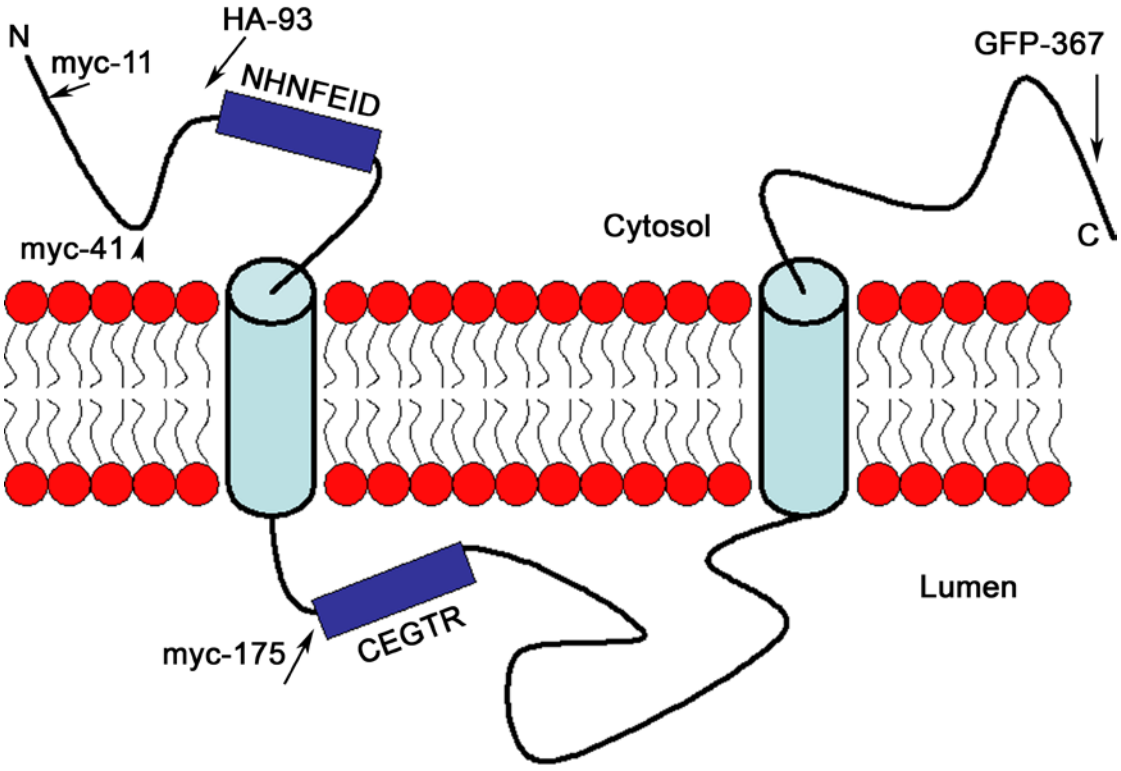
Discussion

The effects of the inhibitor drug CI-976 suggested the importance of LPAT activity in membrane trafficking events including tubule formation, vesicle formation, and Golgi structure maintenance (Drecktrah, Chambers et al. 2003; Chambers and Brown 2004; Brown, Plutner et al. 2008; Yang, Gad et al. 2008). Here, a transmembrane LPAAT, LPAAT3, was identified based on its sequence, cloned into mammalian expression vectors, and found to be localized to Golgi membranes. LPAAT3 is the first Golgi LPAAT to be characterized. Its localization to all regions of the Golgi may indicate a role in membrane trafficking.

Since little is known about LPAAT3, biochemical analyses were done including testing for enzymatic activity, substrate specificity, important catalytic residues, and topology. LPAAT3 has LPAAT activity, which increases the amount of PA in membranes. PA has recently been implicated in multiple trafficking events including the formation of COPI coated vesicles (Yang, Gad et al. 2008). COPI vesicles require acyl-CoA as a co-factor for budding, but the function of the acyl group has been enigmatic (Glick and Rothman 1987; Pfanner, Orci et al. 1989). Perhaps the acyl-CoA is used by an LPAAT enzyme. Although PA is thought to be important for membrane curvature and trafficking, PA can also be a precursor of diacylglycerol (DAG), which can also generate curvature and has similarly been implicated in membrane trafficking from the Golgi and the TGN (Kooijman, Chupin et al. 2005; Fernandez-Ulibarri, Vilella et al. 2007; Asp, Kartberg et al. 2009). In this study, a catalytically inactive form of LPAAT3 was also generated, providing strong evidence for an acid-base catalysis mechanism for LPAAT3.

Based on empirical results, the numbers and locations of transmembrane regions were mapped for LPAAT3 in Figure 2-10. We predict the first conserved motif, NHNFEID to by cytoplasmic while the second motif CEGTR is luminal. Since both of these conserved motifs contain acidic and basic amino acid residues we cannot confidently conclude if LPAAT3 metabolizes phospholipids on the cytoplasmic leaflet of the membrane or the luminal leaflet of the membrane. Although having the two motifs on opposite sides of the membrane was not expected and is quite puzzling, recent data for LPAAT1 topology and activity indicate that LPAAT1 has a similar topology and motif positioning (Yamashita, Nakanishi et al. 2007). In the case of LPAAT1, disruption of either one of the two suspected catalytic motifs completely

Figure 2-10: Model for LPAAT3 topology. Based on independent methods for determining membrane topology, it has been demonstrated that LPAAT3 contains two transmembrane domains. The second transmembrane domain bisects the acyltransferase domains and places each of the conserved catalytic motifs on opposing sides of the membrane. Shown in the diagram are the two conserved motifs that are important for catalytic activity and the locations of epitope tags inserted into LPAAT3 for topology determination.


N
myc-11
HA-93
NHNFEID
GFP-367
Cytosol
C
myc-41
Lumen
myc-175
CEGTR

abolishes activity. We hypothesize that LPAAT3 may require both motifs for either catalysis or substrate binding. LPAAT3 may also have activities on both sides of the membrane such as transferring acyl groups from one leaflet to the other. While the N-terminus of LPAAT3 maps to the cytosolic side of the membrane using these assays, we cannot rule out the possibility that a hydrophobic area within this domain may insert into the membrane, but not as a transmembrane segment.

CHAPTER 3

The Role of LPAAT3 in Maintaining Golgi Structure

Introduction

Proteins that alter the phospholipid makeup of membranes have not only been implicated in membrane trafficking, but also in the maintenance of organelle structure and morphology. For example, overexpression or knockdown of specific phospholipase A_2 enzymes has been shown to fragment the Golgi (Bechler et. al., unpublished data). Additionally, the use of PLA_2 inhibitors like ONO-Rs-082 (ONO) have been shown to cause the Golgi to fragment (de Figueiredo, Polizotto et al. 1999). Golgi membrane tubules are believed to have multiple functions: to form membrane transport compartments and to form membrane bridges between different parts of the Golgi (de Figueiredo, Polizotto et al. 1999). Since PLA_2 enzymes have a role in forming Golgi tubules, altering their expression levels or inhibiting them with a drug disrupts the tubules that keep the Golgi connected.

Inhibition of LPATs with CI-976 causes the Golgi to redistribute the ER, so no changes in Golgi structure could be observed (Drecktrah, Chambers et al. 2003). However, the fact that CI-976 induces Golgi membrane tubules implies a role for LPATs in membrane tubule formation. These same tubules may be important for the maintenance of Golgi architecture. LPAAT3 can alter Golgi membrane phospholipid content and may also be involved in tubule formation. Therefore, LPAAT3 may also have a role in maintaining Golgi structure. In this chapter I will present experiments designed to test this

hypothesis by analyzing the effects of overexpression and reduced expression of LPAAT3 on Golgi morphology.

Materials and Methods

Reagents and Antibodies

ONO-RS-082 (ONO) was purchased from BioMol (Plymouth Meeting, PA) and stored in ethanol at 10 m*M*. The antibody GPP130 (1:1000) was a gift from Adam Linstead (Carnegie Mellon, Pittsburg), hMannII (1:1000) was a gift from Kelly Moreman (Univ. Georgia, Athens), and 10E6 (1:100) and M6PR were previously characterized (Cluett 1993, Brown 1987). All electron microscopy reagents and materials were purchased from EMS (Hatfield, PA).

Immunofluorescence and knockdown

For ONO experiments, HeLa cells were grown on coverslips, washed three times in MEM without serum and incubated with 10 µ*M* ONO for 30 min. For immunofluorescence, cells were processed as previously described in Chapter 2. For knockdown experiments, HeLa cells were grown on 35 mm dishes and transfected with a cocktail of siRNAs designed against LPAAT3 from Dharmacon (Lafayette, CO) using the RNAi MAX transfection kit (Invitrogen, Carlsbad, California) for 72 h. The following siRNA sequences were transfected with their antisense compliments: GAGACCAAGCACCGCGUUAUU, CGUCUUUGCCAGCGGAUCAUU, GCUCCAAGGUCCUGCUAAUU, and GGAAAUAGAAUGACGGGAAUU. Cells were cultured and transfected as described in Chapter 2.

Transmission Electron Microscopy of Thin Sections

Cells were fixed in the dish using glutaraldehyde/osmium tetroxide combination fixative for 2 h (Hirsch and Fedorko 1968). Cells were washed three time for 15 min in 0.1 M sodium cacodylate then stained with 0.25% uranyl acetate for 30 min. Cells were dehydrated using 70%, 95%, and 100% ethanol washes and then removed from the culture dish with propylene oxide. Cells were pelleted in a microcentrifuge and infiltrated with 1:1 propylene oxide and Spurrs for 30 min. The supernatant was removed and replaced with 100% Spurrs overnight. The Spurs was replaced twice more and polymerized at 60°C for 24 h. The polymerized block was sectioned and stained with lead citrate and uranyl acetate. Images were taken using FEI/Philips Morgagni TEM.

Statistics

Error bars on graph represent standard deviation values for a minimum of three hundred cells counted and a minimum of three independent experiments.

Results

LPAAT3 Overexpression Prevents Golgi Breakup by ONO

ONO is a reversible PLA_2 inhibitor that has been shown to block the formation of tubules (de Figueiredo, Polizotto et al. 1999). Cells incubated with ONO do not form BFA-stimulated tubules *in vivo* and isolated Golgi membranes do not form tubules *in vitro*. ONO also causes the Golgi to form multiple mini-stacks instead of the usual compact ribbon structure (de Figueiredo, Polizotto et al. 1999).

Figure 3-1: The effects of LPAAT3 expression on ONO-induced Golgi fragmentation. (a) Cells transfected with LPAAT3 wild type (WT) or LPAAT3 mutant (MT) were treated with 10 μ*M* of the PLA_2 inhibitor ONO for 30 min. Golgi membranes were examined using *trans*-Golgi marker GPP130. All the Golgi membranes appeared fragmented except in cells overexpressing LPAAT3 WT. (b) Quantification of fluorescence results confirms that LPAAT3 WT protects the Golgi against ONO-mediated fragmentation.

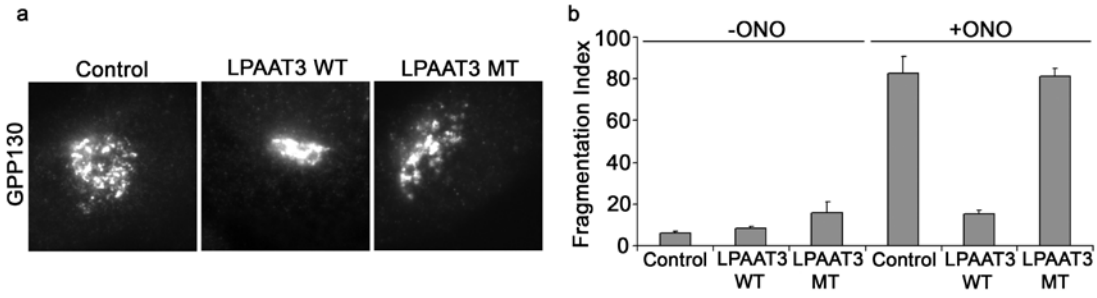
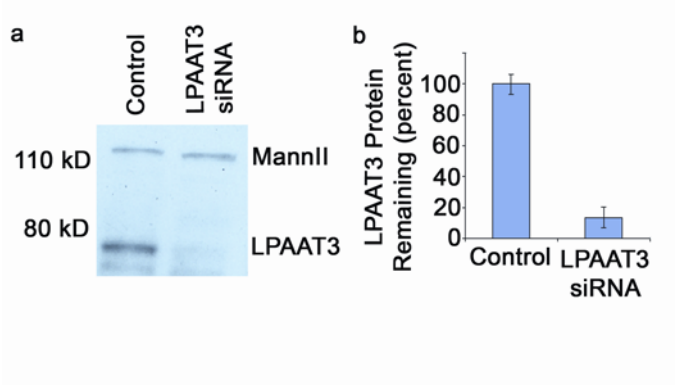

a
Control
LPAAT3 WT
LPAAT3 MT
GPP130
b
-ONO
+ONO
Fragmentation Index
100
80
60
40
20
0
Control
LPAAT3 WT
LPAAT3 MT
Control
LPAAT3 WT
LPAAT3 MT

Figure 3-2: Measuring LPAAT3 knockdown by western blot analysis. (a) A mixture of four siRNA molecules that bind to LPAAT3 mRNA was transfected into cells stably expressing LPAAT3-GFP. Cell lysates were then examined by western blot analysis using antibodies against the resident Golgi enzyme mannosidase II (120 kD) as a loading control and against GFP to determine LPAAT-GFP (72 kD) levels. (b) The bands for LPAAT3-GFP were quantified and indicate that siRNA transfection can reduce overall levels of LPAAT3 by 80-90%.


a
Control
LPAAT3 siRNA
110 kD
80 kD
MannII
LPAAT3
b
LPAAT3 Protein Remaining (percent)
100
80
60
40
20
0
Control
LPAAT3 siRNA

To see if LPAAT3 has any effect on Golgi structure, cells were transfected with LPAAT3 WT and treated with or without 10 μ*M* ONO for 30 min. At that time nearly all of the untransfected control cells showed fragmented Golgi ribbons; however, cells expressing LPAAT3 WT had mostly compact Golgi ribbons (Figure 3-1). Additionally, cells overexpressing the catalytic mutant form of LPAAT3, LPAAT3 MT, showed similar Golgi fragmentation to the control cells (Figure 3-1). This implies that overexpression of LPAAT3 and the production of PA in Golgi membranes can compensate for PLA_2 inhibition, supporting the idea that phospholipids have an important role in determining and maintaining organelle structure.

Knockdown of LPAAT3 Causes Golgi Fragmentation

Since overexpression of LPAAT3 has a protective effect against agents that induce Golgi fragmentation, I tested the effects of reduced expression of LPAAT3, or knockdown, on Golgi morphology. To knockdown LPAAT3, a cocktail of small interfering RNA molecules (siRNA) was transfected into cells stably expressing a GFP-tagged LPAAT3. Cells no longer expressing GFP were assumed to be knocked down for LPAAT3. Additionally, lysates from cells knocked down for LPAAT3 were tested by western blot for the levels of LPAAT3. As shown in Figure 3-2, the siRNA cocktail reduced LPAAT3 levels by 80%. Importantly, reduced expression of LPAAT3 did not have an effect on Golgi proteins because levels of Mann II were unchanged.

Immunofluorescence of the Golgi complex in cells knocked down for LPAAT3 showed that the Golgi was fragmented into multiple puncta (Figure 3-3). Mock transfected and LPAAT3 transfected cells had a Golgi fragmentation

index value of only 10%, while cells knocked down for LPAAT3 had a value of 90% (Figure 3-3b).

LPAAT3 Knockdown Fragments All Golgi Cisternae into Mini-Stacks

Golgi membranes can fragment in different ways. One possibility is for different cisternae to separate, thereby generating clusters of *cis*-Golgi, clusters of *medial*-Golgi, clusters of *trans*-Golgi, and clusters of TGN. Alternatively, fragments can contain elements of each cisterna in the Golgi making each fragment a miniature Golgi stack or "mini-stack."

After siRNA-mediated knockdown of LPAAT3, cells were processed for immunofluorescence against proteins known to be found in different regions of the Golgi. Each of the Golgi cisternae including the *cis*, *medial,* and TGN were observed to be fragmented (Figure 3-4a). This strongly indicated that the fragments formed from LPAAT3 knockdown are Golgi mini-stacks. Further evidence was seen using transmission electron microscopy of thin sections from cells knocked down for LPAAT3. In these cells there are multiple Golgi stacks throughout the cytoplasm of the cell (Figure 3-4b). Control cells typically had only one or two Golgi stacks located centrally in the cells adjacent to the nucleus.

Discussion

Although many cellular components are involved in maintaining Golgi morphology and structure, little is known about the role of phospholipids themselves in maintaining the stacked and centralized structure. Many of the factors that disrupt Golgi structure are also involved in proper Golgi functions

Figure 3-3: Golgi complex fragmentation by LPAAT3 knockdown. (a) HeLa cells stably expressing LPAAT3-GFP were transfected with siRNA molecules targeting LPAAT3 mRNA. In cells with reduced GFP fluorescence, LPAAT3 was presumed knockdown. These cells contained fragmented Golgi. (b) Quantification verified that LPAAT3 knockdown dramatically increases the Golgi fragmentation index.

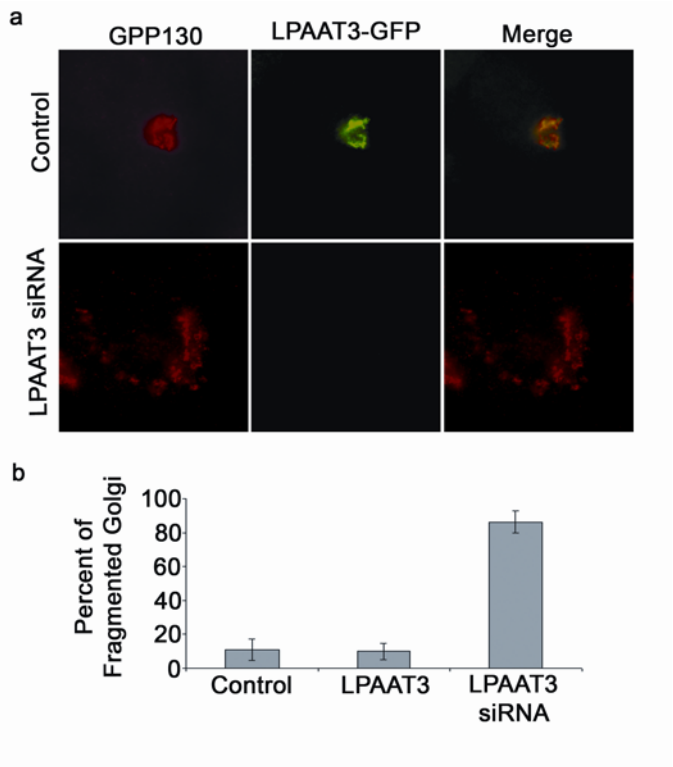
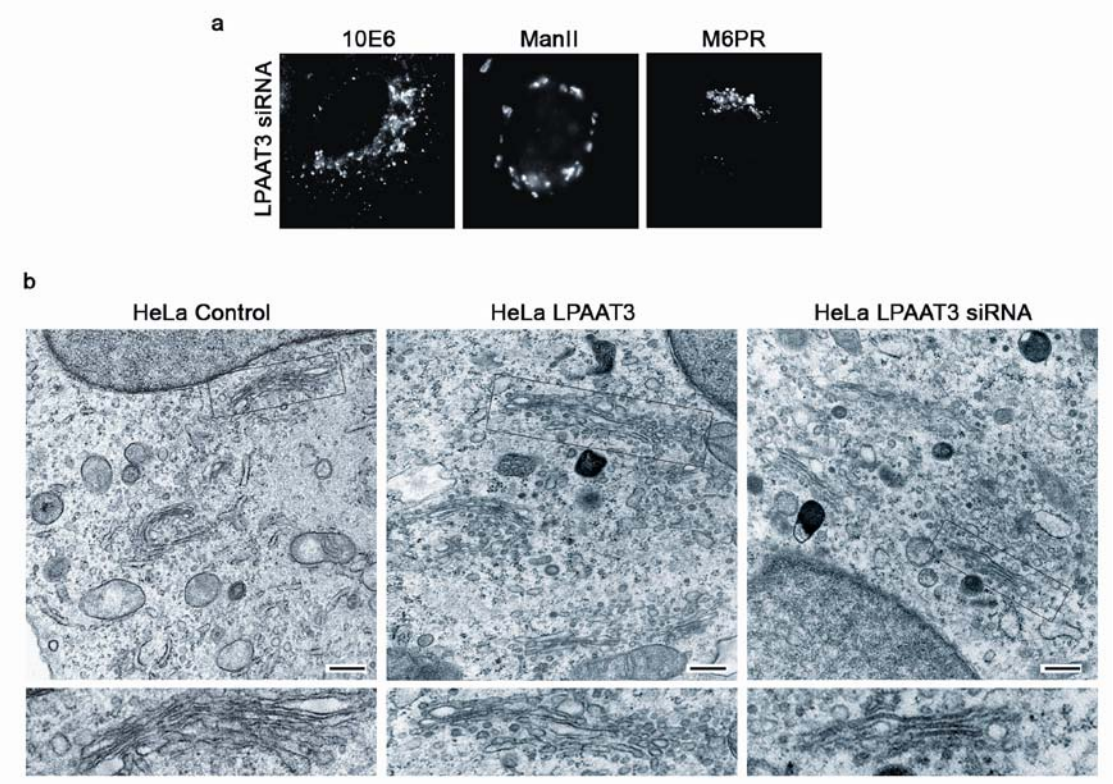

a
GPP130
LPAAT3-GFP
Merge
Control
LPAAT3 siRNA
b
Percent of Fragmented Golgi
100
80
60
40
20
0
Control
LPAAT3
LPAAT3 siRNA

Figure 3-4: LPAAT3 knockdown results in Golgi mini-stacks. (a) Cells knockdown for LPAAT3 have Golgi fragments that contain proteins normally found in the *cis*-Golgi (10E6), the *medial*-Golgi (ManII), and the Trans Golgi Network (M6PR). (b) Electron micrographs of LPAAT3 knockdown cells contain multiple in-tact Golgi stacks indicating that the Golgi fragments observed by fluorescence are mini-stacks.


a
10E6
ManII
M6PR
LPAAT3 siRNA
b
HeLa Control
HeLa LPAAT3
HeLa LPAAT3 siRNA

such as the regulation of secretion and the modification of newly synthesized proteins.

As shown in Chapter 2, LPAAT3 generates PA and uses LPA in the Golgi membranes of a cell. Overexpression or knockdown of LPAAT3 causes the phospholipid make-up of the membrane to change. This appears to have a profound effect on the morphology and the overall structure of the Golgi. Overexpression protects the Golgi from fragmentation-inducing agents such as ONO while knockdown causes fragmentation into Golgi mini-stacks. Knockdown of LPAAT3 may allow PLA_2 enzymes to produce excess lysophospholipids (LPL). These LPLs are thought to produce membrane tubules, and excessive tubules could fragment the Golgi. If LPAAT3 has an effect on Golgi structure, it may also have an effect on Golgi function, specifically membrane trafficking from the Golgi to the ER and trafficking out of the Golgi to the plasma membrane.

CHAPTER 4

LPAAT3 has a Role in Membrane Tubule Formation and Golgi Trafficking

Introduction

Phospholipid-modifying enzymes including PLA_2 enzymes and LPAATs have been shown to either positively promote the formation of membrane tubules or negatively regulate their formation, respectively (Brown, Chambers et al. 2003). This was discovered using inhibitors against those two classes of enzymes. The PLA_2 inhibitors ONO and BEL (HELSS) prevent Golgi tubules from forming *in vitro* and *in vivo* (de Figueiredo, Drecktrah et al. 1998). Those inhibitors can also prevent Golgi tubules from forming when cells are treated with the fungal metabolite brefeldin A (BFA). BFA has been shown to rapidly cause the Golgi to tubulate and redistribute enzymes and lipids to the ER. Another antagonist that also results in Golgi tubules is the LPAT inhibitor CI-976 (Drecktrah, Chambers et al. 2003). This pharmacological agent revealed how important LPAATs are for trafficking and was the impetus to study LPAATs.

Membrane tubules are not only formed in the presence of BFA and CI-976; they are involved in the constitutive recycling of many proteins that escape the ER. One protein that continuously cycles between the ER and the Golgi in tubules is the ERGIC53/p58 receptor protein, which is involved in the trafficking of glycosylated proteins (Klumperman, Schweizer et al. 1998). ERGIC-53/p58 is therefore a useful tool for observing the bidirectional transport pathways that exist between the ER and the Golgi. A very useful *in vitro* Golgi tubulation assay has been developed that only requires isolated

Golgi membranes and a preparation of bovine brain cytosol (BBC) to induce tubules outside of the cellular environment (Banta, Polizotto et al. 1995).

While CI-976 has shown the importance for LPATs and Golgi tubules in ER to Golgi (Brown, Plutner et al. 2008) and Golgi to ER transport (Drecktrah, Chambers et al. 2003), less is known about anterograde trafficking from the Golgi to the plasma membrane. Since CI-976 results in the Golgi redistributing back to the ER, the LPAAT antagonist has only limited use for studying trafficking from the Golgi or TGN to the plasma membrane. Since LPAAT3 is partially localized to the Golgi, has LPAAT activity, and has an effect on Golgi structure, it may also have a role in Golgi trafficking. Here I examine Golgi trafficking, *in vivo* and *in vitro* as well as retrograde and anterograde trafficking under the conditions of LPAAT3 overexpression or knockdown.

Materials and Methods

Transfection and Immunofluorescence

The following antibodies were used: GPP130 was used at 1:1000 dilution, ERGIC-53 was used at 1:100 dilution, and β-COP was used at 1:50 dilution. Cells were transfected with pEDsRed N-1 ts045 VSV-G, pEGFP N-1 ssHRP-FLAG, pEGFP N-1 LPAAT3 Wild Type, pEFGP N-1 LPAAT3 Mutant, and pEGFP N-1 LPAAT6 Wild type using Fugene 6 (Roche). LPAAT3 was knocked down as described in Chapter 3.

Tissue culture cells were grown and maintained as described in Chapter 2. For BFA experiments, cells were washed with MEM (no serum) and treated with 5 µg/ml BFA in DMSO at either 37°C or 24°C. For CI-976 experiments, cells were washed with MEM (no serum) and treated with 50 µ*M*

CI-976 in DMSO. For ERGIC-53 trafficking, cells were shifted to a 15°C water bath for 3 h then quickly warmed by changing the medium to 37°C. For ts045 VSV-G experiments, cells were incubated at 40°C for 18 h and then shifted to 32°C. Coverslips containing cells were fixed and processed for immunofluorescence as described in Chapter 3.

VSVG/ssHRP

Cells were transfected with pEGFP N-1 ssHRP-Flag for 24 h. Cell medium was removed and the cells were washed three times and replaced with fresh media. To sample HRP activity in the media, 50 µL of media was removed from the culture dishes for every time point and to measure non-secreted HRP activity, the cells were scraped from the dishes, incubated in PBS +1% NP-40 for 10 min, and centrifuged at max speed to remove cell debris. For each sample, 10 µL was incubated with 50 µL of TMB reagent (Sigma, St. Louis, MO). When samples turned blue, 50 µL of 1 *M* HCl was added to stop the reaction. All reactions were done in duplicate and measured using the SpectraMax 190 (Molecular Devices, Sunnyvale, CA) 96-well plate reader at 450 nm. To measure ts045 VSV-G DsRed localization, 100 images from fixed cells were processed using ImageJ (NIH) to measure the pixel intensity in the Golgi and total cell regions of interest (ROI). Background values of pixel intensity per unit area were measured and subtracted from each ROI.

Sholl Analysis

A modified Sholl analysis (Sholl 1953) was performed to quantify the number and length of Golgi membrane tubules in cells expressing

fluorescence Golgi markers. Digital micrographs were traced in NIH ImageJ and the intersections of those tubules with concentric circles were determined using the Sholl software plug-in. These values were used to determine the number of tubules at each particular length.

In vitro tubulation

Golgi complexes were enriched as indicated above. Isolated membranes (Chapter 2) were incubated with either 1.4 mg/ml BBC or 1.4 mg/ml BSA in tubulation buffer (0.1 m*M* ATP, 50 m*M* KCl, 1 m*M* MgCl2, 25 m*M* Tris-Cl, 10 m*M* HEPES, pH 7.4) for 15 min at 37°C. Bovine brain cytosol (BBC) was prepared as described previously (Banta, Polizotto et al. 1995). Reactions were placed on ice and then on 300 mesh copper EM grids coated with Formvar and carbon. Golgi membranes were negatively stained with 2% phosphotungstic acid (pH 7.2) viewed using the Morgagni 286 electron microscope, and quantified as tubulated or not tubulated.

Results

LPAAT3 Overexpression Slows, and Knockdown Accelerates the Kinetics of Golgi to ER Retrograde Trafficking

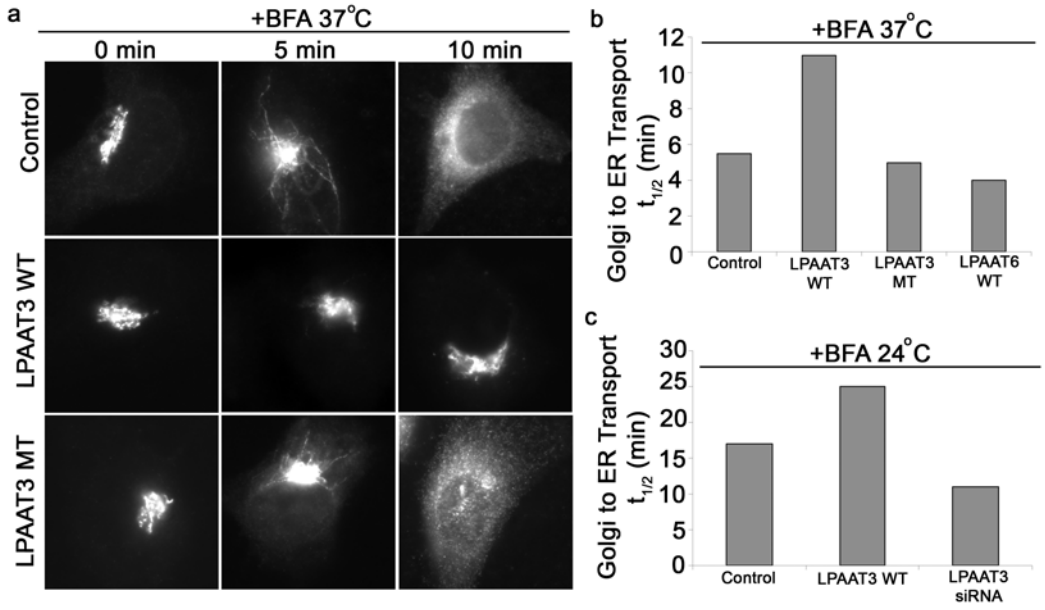
The Effects of LPAAT3 Expression on BFA Tubules and Retrograde Trafficking

Since the LPAT inhibitor CI-976 causes Golgi membranes to form membrane tubules, overexpression of an LPAAT is expected to have a negative impact on membrane tubule biogenesis. Membrane tubules are sometimes difficult to observe under normal conditions. Therefore, BFA was

used to induce rapid tubule formation. HeLa cells and cells transfected with either LPAAT3 WT, LPAAT3 MT, LPAAT3 knockdown, or LPAAT6 as a control were treated with BFA for various time points and then fixed and stained for Golgi markers (Figure 4-1). It was observed that cells transfected with LPAAT3 did not readily form Golgi tubules and took longer to fuse with the ER. Conversely, cells knocked down for LPAAT3 formed tubules much faster, and the Golgi fused with the ER very quickly. Tubules formed so quickly that the experiment was repeated at 24°C instead of the usual 37°C to slow the process down so LPAAT3 knockdown cells could be fixed and counted; however, the results were the same (Fig. 4-1c).

Figure 4-1c shows that the Golgi in cells knocked down for LPAAT3 formed tubules faster and fused with the ER faster than controls and that cells overexpressing LPAAT3 WT formed tubules much more slowly. Cells with compact Golgi, tubulated Golgi, or diffuse Golgi/ER were quantified (not shown). The $t_{½}$ for each condition was calculated and shown in Figure 4-1b and 4-1c for cells incubated at 37°C and 24°C respectively. For cells overexpressing LPAAT3 and treated with BFA at 37°C, the Golgi took twice as long to go from a compact Golgi to diffuse ER while the LPAAT6 and LPAAT3 MT was similar to control cells. A similar result was seen at 24°C. Conversely, LPAAT3 knockdown cells were 35% faster than control cells. This may indicate a role for LPAAT3 expression and membrane tubule formation.

Figure 4-1: The effects of LPAAT3 expression on BFA-stimulated Golgi tubules. (a) Control cells or cells transfected with LPAAT3 wild type (WT), LPAAT3 mutant (MT), LPAAT6, or LPAAT3 siRNA were treated with 10 µg/ml brefeldin A (BFA) for various lengths of time at either 37°C. (b) At 37°C LPAAT3 WT, but not the others, slowed the formation of BFA-stimulated tubules and retrograde trafficking as measured by the time it took for the Golgi to become diffuse. (c) At 24°C, LPAAT3 knockdown cells were faster at forming BFA-stimulated Golgi tubules and retrograde trafficking. Knockdown cells were done at 24°C because tubules formed too quickly at 37°C to quantify.


a
+BFA 37°C
0 min
5 min
10 min
Control
LPAAT3 WT
LPAAT3 MT
b
+BFA 37°C
Golgi to ER Transport
t1/2 (min)
12
10
8
6
4
2
0
Control
LPAAT3 WT
LPAAT3 MT
LPAAT6 WT
c
+BFA 24°C
Golgi to ER Transport
t1/2 (min)
30
25
20
15
10
5
0
Control
LPAAT3 WT
LPAAT3 siRNA

The Effects of LPAAT3 Expression on CI-976-Induced Golgi Tubules

Although BFA is useful for looking at Golgi membrane tubules, it disrupts other trafficking components. Figure 4-2 shows that cells treated with BFA have less COPI coat protein located on Golgi membranes as expected. Although LPAAT3 expression levels did not effect β-COP (Figure 4-2) or Arf1 (not shown) on the Golgi, there may be other unforeseen consequences of using BFA. To address this, CI-976 was also used to generate Golgi membrane tubules.

CI-976 is an LPAT inhibitor and only alters phospholipid metabolism without disrupting other trafficking components. Because CI-976 inhibits a BFA-independent pathway, it is useful for testing the effects of LPAAT3 expression on Golgi tubules. Figure 4-3 shows images of cells treated with CI-976 and demonstrates how cells overexpressing LPAAT3 do not form tubules as quickly as control cells and LPAAT3 MT cells. In contrast, cells knocked down for LPAAT3 form those tubules much faster. The graph in Figure 4-3b shows the rate of Golgi to ER retrograde transport in the cells treated with CI-976.

The Effects of LPAAT3 Expression on ERGIC-53/p58 Trafficking

One last means of measuring Golgi to ER transport is to use a system that is independent of both pharmacological agents and transfected markers. For this, the endogenous ERGIC-53/p58 was used to monitor Golgi to ER transport. ERGIC-53/p58 is a transport receptor that is located in the ER and the early Golgi compartment called the ERGIC (Klumperman, Schweizer et al. 1998). ERGIC-53/p58 exits the ER in COPII coated vesicles with secretory cargo and returns in Golgi tubules. A useful feature is that at 15°C,

Figure 4-2: Dissociation of Arf1 and β-COP by short-term BFA treatment. In HeLa cells, LPAAT3-GFP stable cells, LPAAT3-GFP wild type (WT) transfected cells, and LPAAT3-GFP mutant (MT) transfected cells, the COPI coat protein β-COP remained associated with the Golgi membranes. After BFA was added for 2 min to all cells, β-COP became cytoplasmic with no change to LPAAT3 localization.

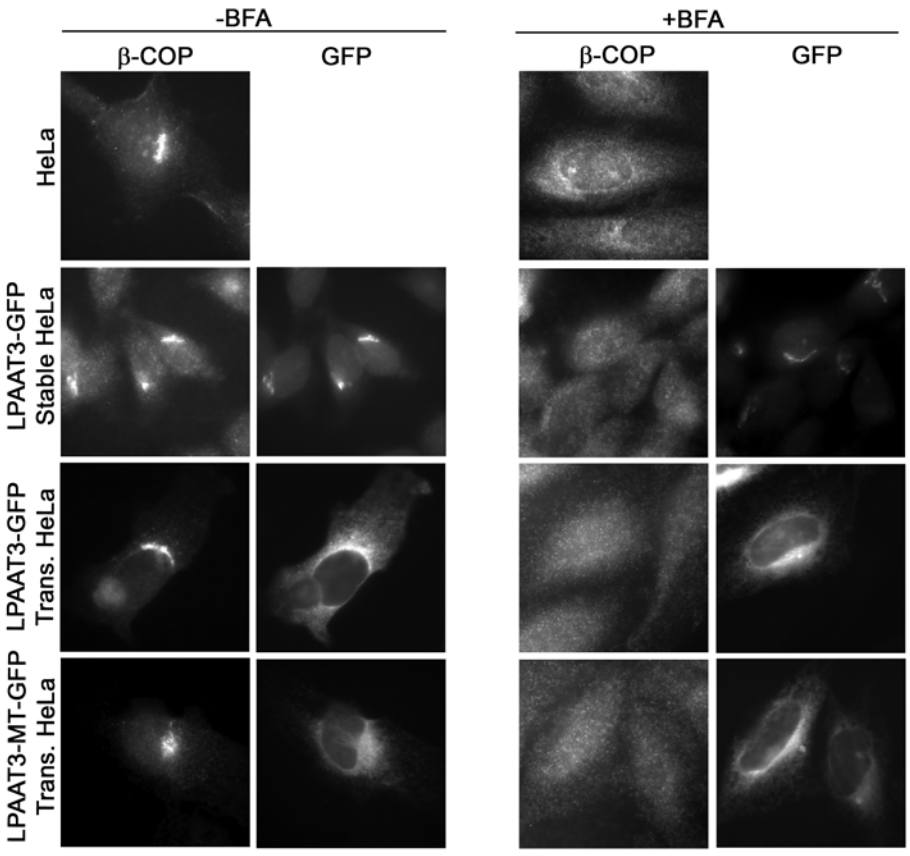
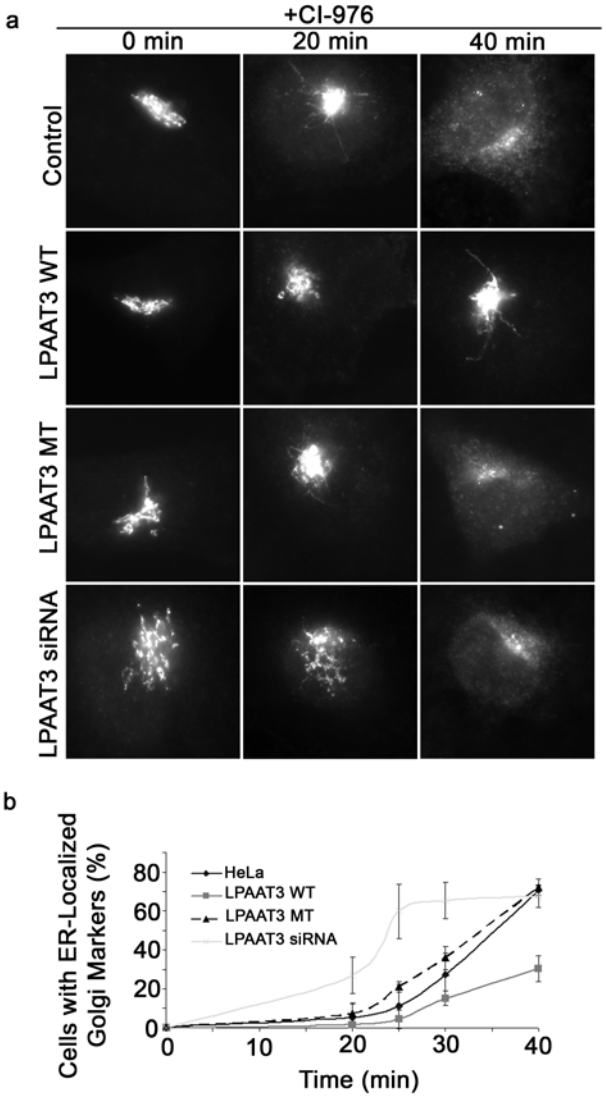

-BFA
+BFA
β-COP
GFP
β-COP
GFP
HeLa
LPAAT3-GFP Stable HeLa
LPAAT3-GFP Trans. HeLa
LPAAT3-MT-GFP Trans. HeLa

Figure 4-3: The effects of LPAAT3 expression on CI-976-stimulated Golgi tubules. (a) Cells transfected with LPAAT3-GFP wild type (WT), LPAAT3-GFP mutant (MT), LPAAT3 siRNA, and control cells were treated with 50 µ*M* CI-976 for various amounts of time. Cells overexpressing LPAAT3-GFP WT were slower to form Golgi tubules than control and LPAAT3-GFP MT overexpressing cells, while cells knocked down for LPAAT3 were faster at forming Golgi tubules. (b) Quantification of a time course of CI-976 treatment of cells overexpressing LPAAT3 compared to cells knockdown for LPAAT3. Cells were scored for the Golgi marker GPP130 becoming diffuse/ER.


a
+CI-976
0 min
20 min
40 min
Control
LPAAT3 WT
LPAAT3 MT
LPAAT3 siRNA
b
Cells with ER-Localized Golgi Markers (%)
HeLa
LPAAT3 WT
LPAAT3 MT
LPAAT3 siRNA
0
20
40
60
80
0
10
20
30
40
Time (min)

ERGIC53/p58 is able to exit the ER, but not return, so it accumulates in the ERGIC compartment until the temperature is changed. Shifting back to 37°C results in a near-synchronous wave of ERGIC53/p58 transport from the *cis*-Golgi to the ER via membrane tubules (Itin, Schindler et al. 1995; Klumperman, Schweizer et al. 1998).

Cells expressing LPAAT3 WT or knocked down for LPAAT3 were incubated at 15°C, shifted to 37°C and fixed to observe the rate of ERGIC to ER trafficking (Figure 4-4a). After only two minutes, 60% of the ERGIC-53 protein was back to the ER in LPAAT3 knockdown cells while in control cells only 40% had returned. In cells overexpressing LPAAT3, less then 10% had returned (Figure 4-4b). These data, taken together with the BFA and CI-976 observations, indicate that overexpression of LPAAT3 significantly slows membrane tubule-mediated retrograde trafficking. In contrast, knockdown of LPAAT3 has the opposite effect and accelerates the rate of retrograde trafficking.

LPAAT3 Expression Effects Anterograde Trafficking from the TGN to the Plasma Membrane

Membrane tubules and vesicles are also involved in anterograde transport from the Golgi, specifically from the TGN (Bard and Malhotra 2006). Historically, the vesicular stomatitis virus G protein (VSV-G) has been used as a marker for transport through the secretory pathway. Cells overexpressing LPAAT3 or knocked down for LPAAT3 were co- transfected with ts045 VSV-G-DsRed. At 40°C ts045 VSV-G-DsRed cannot properly fold and remains trapped in the ER (Fig. 4-5). After the cells were shifted to 32°C, the ts045 VSV-G-DsRed folded and was transported to the Golgi as expected.

Figure 4-4: ERGIC-53/p58 retrograde trafficking and LPAAT3 expression. (a) Clone 9 rat hepatocytes, Clone 9 cells stably expressing LPAAT-GFP, and Clone 9 cells transfected with LPAAT3 siRNA were incubated at 15°C for 3 h to accumulate the ERGIC-53/p58 protein in the cis-Golgi. Cells were then warmed to 37°C to allow retrograde trafficking of ERGIC-53/p58. Cells with LPAAT3-GFP maintained the *cis*-Golgi localization of ERGIC-53/p58 as shown by immunofluorescence. Cells knocked down for LPAAT3 rapidly transported ERGIC-53/p58 to the ER compared to control. Quantification of cells with ER-localized ERGIC-53/p58 at 2 min (b) and 5 min (c) showed that ERGIC-53/p58 arrived at the ER faster in knockdown cells and slower in LPAAT3-GFP cells.

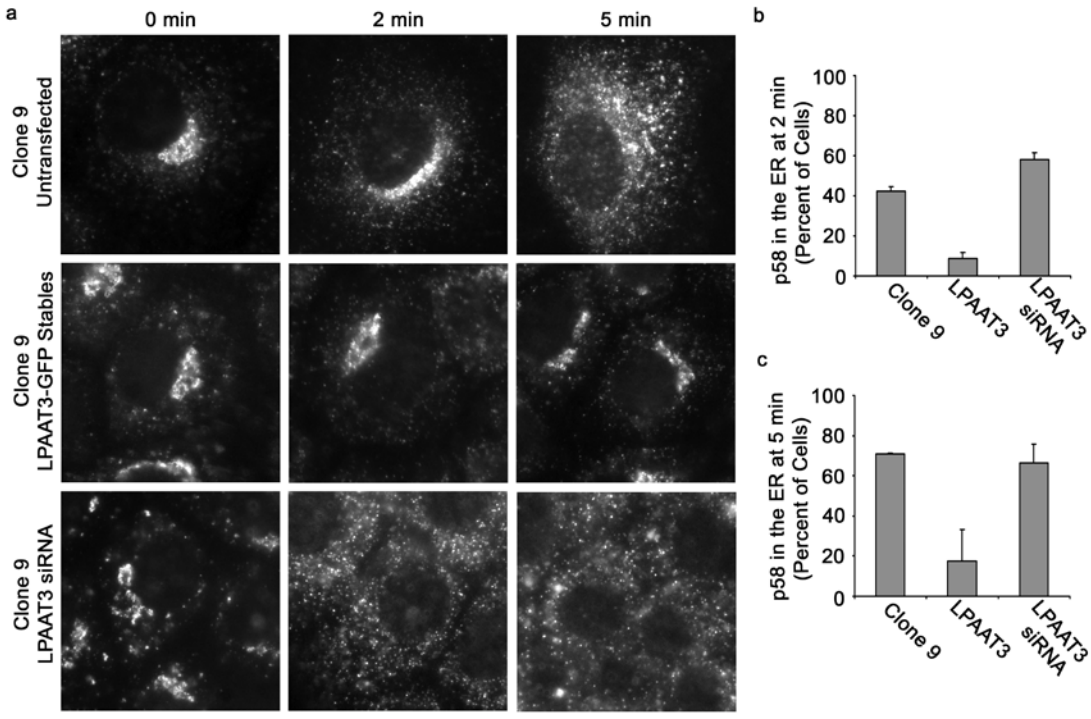

a
0 min
2 min
5 min
Clone 9
Untransfected
Clone 9
LPAAT3-GFP Stables
Clone 9
LPAAT3 siRNA
b
p58 in the ER at 2 min
(Percent of Cells)
100
80
60
40
20
0
Clone 9
LPAAT3
LPAAT3
siRNA
c
p58 in the ER at 5 min
(Percent of Cells)
100
80
60
40
20
0
Clone 9
LPAAT3
LPAAT3
siRNA

Figure 4-5: LPAAT3 expression and ts045 VSV-G-DsRed trafficking from the TGN to the cell surface. (a) HeLa cells overexpressing LPAAT3-GFP WT or knocked down for LPAAT3 were transfected with ts045 VSV-G-DsRed and incubated at 40°C for 18 h. Cells were then shifted to 32°C for various lengths of time. The VSV-G protein easily arrived at the Golgi in all cells, but only efficiently trafficked to the cell surface in control cells. (b) By measuring the fluorescence in the Golgi and comparing that to total cell fluorescence, cells either overexpressing or knocked down for LPAAT3 were shown to be deficient in ts045 VSV-G-DsRed trafficking out of the Golgi.

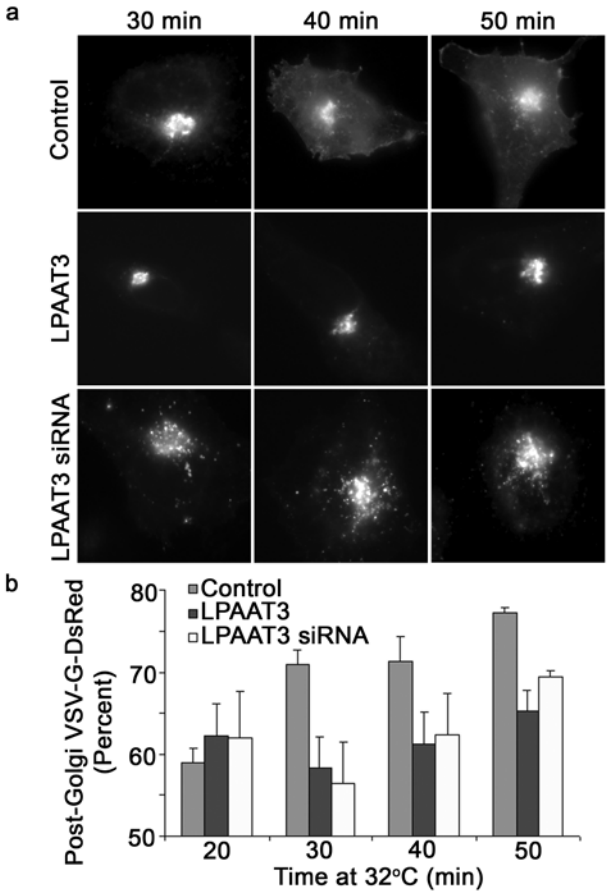
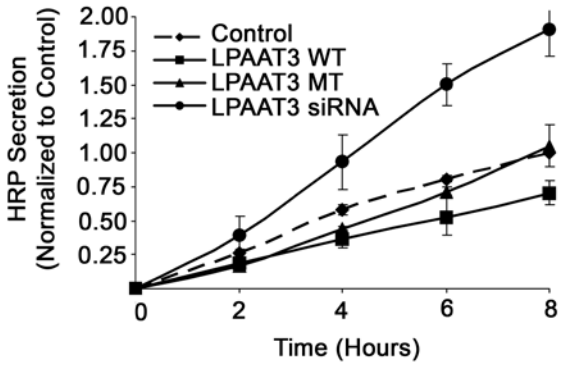

a
30 min
40 min
50 min
Control
LPAAT3
LPAAT3 siRNA
b
Control
LPAAT3
LPAAT3 siRNA
Post-Golgi VSV-G-DsRed (Percent)
80
70
60
50
20
30
40
50
Time at 32°C (min)

Figure 4-6: Secretion of ssHRP is altered by LPAAT3 expression. Cells expressing LPAAT3-GFP wild type (WT), LPAAT3-GFP mutant (MT), LPAAT3 siRNA, and control cells were transfected with an ssHRP-FLAG expression vector. ssHRP was made in the ER, transported to the extracellular space, and measured by taking media samples. LPAAT3 knockdown cells secreted ssHRP twice as fast as control cells and LPAAT3 MT cells while LPAAT3 wild type cells were much slower.


HRP Secretion
(Normalized to Control)
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0
2
4
6
8
Time (Hours)
Control
LPAAT3 WT
LPAAT3 MT
LPAAT3 siRNA

However, at later time points when the ts045 VSV-G-DsRed was expected to transport from the Golgi to the plasma membrane, accumulation of ts045 VSV-G-DsRed was seen in cells overexpressing LPAAT3 and knockdown for LPAAT3 (Figure 4-5a). The percent of fluorescence in the Golgi was compared to total fluorescence in Figure 4-5b to quantify TGN to plasma membrane transport.

While the transmembrane VSV-G is packaged into tubules at the TGN, soluble cargo can exit the Golgi through multiple pathways (Bard and Malhotra 2006). A secretable form of horseradish peroxidase (ssHRP) is a soluble protein that uses the secretory pathway and is easily detectable by enzymatic assay once released from the cell. Cells expressing ssHRP were tested to see if LPAAT3 expression has any effect on secretion. ssHRP in the extracellular media were collected and measured after various time points. Cells overexpressing LPAAT3 were slower at secreting ssHRP while cells knocked down for LPAAT3 were much faster (Figure 4-6). LPAAT3 MT had little effect on ssHRP secretion. Both VSV-G and ssHRP anterograde trafficking appear to be affected by LPAAT3, which implies a role for LPAAT3 and phosphatidic acid in both retrograde and anterograde Golgi trafficking.

LPAAT3 Negatively Regulates Golgi Membrane Tubule Biogenesis

The Golgi complex transports proteins to the ER through the retrograde pathway and to the plasma membrane through the anterograde pathway, but the actual transport cargo can be sorted into either coated vesicles or membrane tubules (Bard and Malhotra 2006). Many of the transport pathways discussed so far, including BFA-induced, ERGIC-53, and VSV-G, use membrane tubules, so LPAAT3 may be involved in regulating membrane

tubule formation. Images from cells in the BFA assays were re-examined for the rate of Golgi to ER transport and for the extent of Golgi membrane tubules. The Sholl analysis, which has been used previously to measure dendrite branching in neurons (Sholl 1953), was applied to these images as a means of measuring both the number and length of Golgi membrane tubules. Figure 4-7a shows that cells overexpressing LPAAT3 have far fewer tubules when treated with BFA compared to control cells. Also, those tubules are far shorter than those in control cells (Figure 4-7b). This novel method for quantification was useful for determining the extent of Golgi tubules in these cells.

Another approach for looking at Golgi tubules is an *in vitro* method. Here, cells stably expressing LPAAT3 were processed to isolate Golgi membranes. These Golgi membranes were incubated with bovine brain cytosol (BBC), which has been shown to induce tubules in a PLA_2-dependent manner (Cluett, Wood et al. 1993; de Figueiredo, Drecktrah et al. 1998). Many more of the Golgi membranes from control cells formed tubules *in vitro* compared to those from LPAAT3 overexpressing cells (Figure 4-8). These data indicate that LPAAT3 negatively regulates Golgi membrane tubule formation.

Membrane tubules may either function independently as transport carriers for cargo, or may act as a membranous scaffold for vesicle formation. To test if LPAAT3 has a role in the formation of vesicles as well as membrane tubules, an *in vitro* COPI budding assay was used. Golgi membranes were incubated with rat liver cytosol and allowed to form COPI vesicles, which were separated by sucrose-gradients and high-speed centrifugation. Figure 4-9 shows that these assays have high variability and that only a slight decrease

Figure 4-7: Golgi tubule number and length is altered by LPAAT3 overexpression. Control cells and cells overexpressing LPAAT3-GFP wild type were treated with 10 µg/mL BFA for various lengths of time. Images of the cells were then processed using the Sholl Analysis to measure membrane tubule length and number. (a) Cells overexpressing LPAAT3 had fewer Golgi tubules, especially at early time points following BFA addition. (b) Moreover, cells overexpressing LPAAT3 also have shorter membrane tubules. The Sholl Analyses were aided by the efforts of Griselda Yvone, a former undergraduate in the lab.

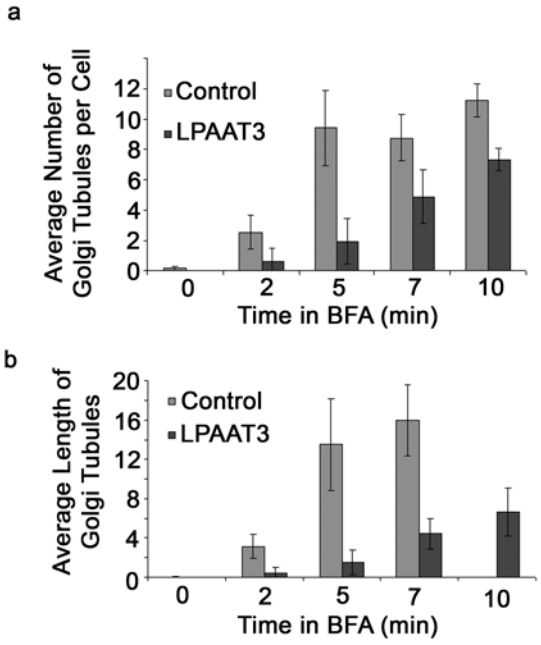
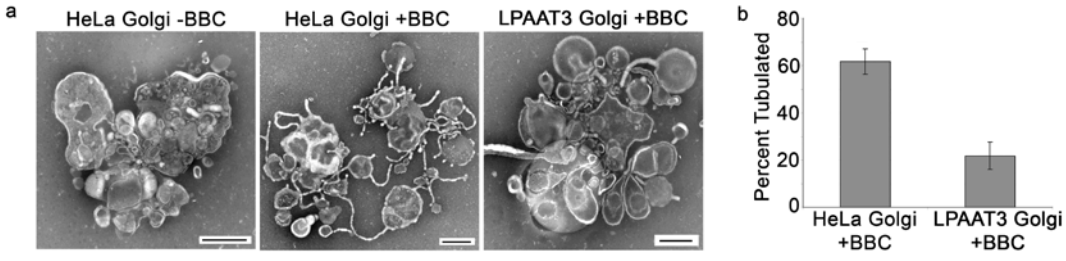

a
Average Number of Golgi Tubules per Cell
Control
LPAAT3
0
2
4
6
8
10
12
0
2
5
7
10
Time in BFA (min)
b
Average Length of Golgi Tubules
Control
LPAAT3
0
4
8
12
16
20
0
2
5
7
10
Time in BFA (min)

Figure 4-8: *In vitro* tubulation assay. (a) Golgi membranes were extracted from HeLa cells and HeLa cells stably expressing LPAAT3. These Golgi membranes were then incubated with 1.6 mg/mL bovine brain cytosol (BBC) to stimulate membrane tubule formation. (b) LPAAT3 Golgi formed far fewer tubules than control Golgi.


a
HeLa Golgi -BBC
HeLa Golgi +BBC
LPAAT3 Golgi +BBC
b
Percent Tubulated
80
60
40
20
0
HeLa Golgi +BBC
LPAAT3 Golgi +BBC

in COPI vesicles was observed in cells stably expressing LPAAT3 versus control cells.

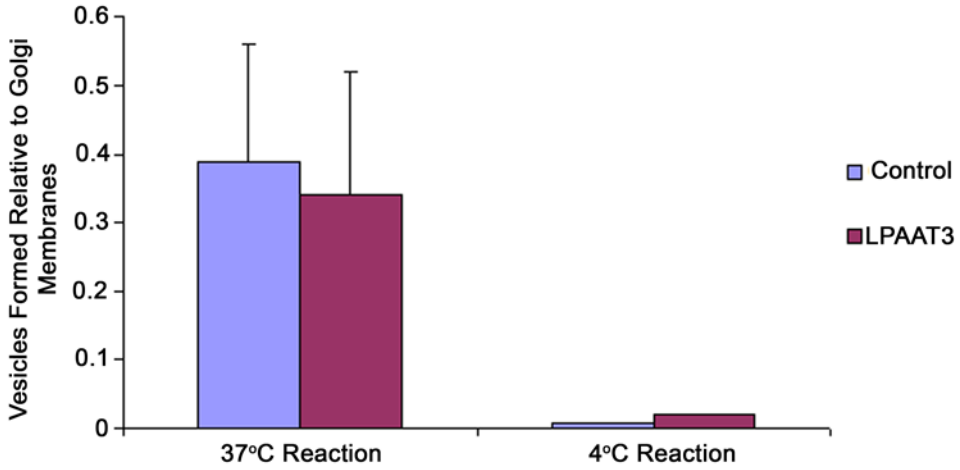
Discussion

The LPAT inhibitor CI-976 inhibits the formation of COPI and COPII coated vesicles and rapidly causes the Golgi to form membrane tubules which redistribute the Golgi to the ER (Drecktrah, Chambers et al. 2003; Brown and Schmidt 2005; Brown, Plutner et al. 2008; Yang, Gad et al. 2008). This implies that LPAAT proteins may have roles in multiple membrane trafficking events by altering relative amounts of lysophospholipids and phospholipids. Here I examined the potential roles of LPAAT3 in Golgi membrane trafficking by overexpressing LPAAT3 or by knocking down expression of LPAAT3.

Overexpression of LPAAT3 slowed retrograde Golgi to ER trafficking when membrane tubules were stimulated by the addition of BFA or CI-976. Furthermore ERGIC to ER trafficking was also slowed for the endogenous protein ERGIC-53/p58. Additionally, knocking down LPAAT3 had the opposite effect and accelerated retrograde transport. The fact the LPAAT3 catalytic mutant had little effect on the kinetics of Golgi to ER transport implies that the LPAAT activity of LPAAT3 is critical for these phenotypes and that the phospholipids in the membrane are therefore important factors in the formation of transport intermediates.

LPAAT3 overexpression also slowed anterograde transport of both VSV-G (transmembrane) and ssHRP (soluble) cargo proteins. Cells knocked down for LPAAT3 have accelerated anterograde trafficking for ssHRP, although it is still unclear why VSV-G transport was not accelerated. One hypothesis is that VSV-G is transmembrane cargo and is transported from the

Figure 4-9: *In vitro* COPI vesicle budding assay. Golgi membranes from HeLa cells and cells stably expressing LPAAT3 were incubated with rat liver cytosol at 37°C to produce vesicles and 4°C as a control. Golgi membranes and vesicles were then separated by sucrose-gradient high-speed centrifugation and quantified by western blot against the mannoside II protein. (a) The relative number of vesicles formed by control cells and cells expressing LPAAT3 are about the same.


Vesicles Formed Relative to Golgi Membranes
0.6
0.5
0.4
0.3
0.2
0.1
0
37°C Reaction
4°C Reaction
Control
LPAAT3

TGN to the basolateral region of the plasma membrane while ssHRP is soluble, and therefore can be transported more freely to any region of the plasma membrane. Cells knocked down for LPAAT3 may accelerate one pathway (soluble/apical) while slowing another (transmembrane/basolateral).

The Sholl Analysis, the *in vitro* tubulation assay, and the COPI budding assay indicate specific roles for LPAAT3 in negatively regulating Golgi membrane tubules. These data support previously published data regarding PLA_2 enzymes and tubule biogenesis as well as data from CI-976 experiments. Without LPAAT activity in the Golgi, membrane tubules may form without regulation, and therefore cause changes to Golgi morphology and the rate of Golgi trafficking. If Golgi trafficking is too fast, proteins may not be properly modified before exiting the Golgi and may be transported to the wrong destination. Conversely, if trafficking is too slow, like in cells overexpressing LPAAT3, cell growth can be diminished since new membrane cannot quickly arrive at the plasma membrane. Cell migration and efficient protein synthesis may also be adversely affected.

CHAPTER 5

The Role of Phospholipase A_2 Activity in Trafficking from the Trans Golgi Network (TGN) to the Plasma Membrane

Introduction

Cargo exiting the TGN is targeted to the basolateral or apical plasma membrane, and is concentrated in coated vesicles or membrane tubules. Some protein cargo, including VSV-G and collagen, is found mainly in TGN-derived membrane tubules (Hirschberg, Miller et al. 1998; Polishchuk, Di Pentima et al. 2003). The mechanisms of how cargo is sorted into either tubules or coated vesicles, and how that cargo is targeted to endosomes or the plasma membrane, is largely unknown. The current model for TGN tubule formation is that membrane domains in the TGN become enriched in transport cargo, but exclude resident TGN proteins (Bard and Malhotra 2006). Membrane tubules are pulled from these domains with the help of kinesin, and then undergo fission (Polishchuk, Di Pentima et al. 2003). Some of the factors involved in fission include heterotrimeric G proteins and protein kinase D (PKD) (Yeaman, Ayala et al. 2004; Diaz Anel and Malhotra 2005). When overexpressed, the kinase inactive form of PKD (PKD-KD) leads to an extensive network of tubules forming from the TGN, but not the Golgi, which are thought to form as a result of fission inhibition (Liljedahl, Maeda et al. 2001; Maeda, Beznoussenko et al. 2001).

Fission of TGN secretory membrane tubules requires many factors including the phospholipids within the membrane itself. The metabolism of phosphatidic acid (PA), diacylglycerol (DAG), and phosphotidylinositol (PI) is thought to have roles in tubule fission (Bard and Malhotra 2006). PKD itself

binds to DAG, which may act as a binding domain for the fission machinery (Maeda, Beznoussenko et al. 2001; Baron and Malhotra 2002). Phospholipids are also thought to generate unstable domains within the membrane that promote hemi-fission and eventual membrane fission by altering the curvature and physical properties of the membrane itself (Bard and Malhotra 2006). Although a great deal is known about how TGN transport carriers separate from the donor membrane, little is known about how these extensive TGN tubules form. Previous studies have suggested that phospholipids are important in regulating membrane tubule formation as well as tubule fission (Brown, Chambers et al. 2003).

Phospholipase A_2 (PLA_2) enzymes generate lysophospholipids from phospholipids in cellular membranes (Brown, Chambers et al. 2003). PLA_2 enzymes have been shown to have a role in the formation of Golgi retrograde tubules, intra-Golgi tubules, and recycling endosome tubules both *in vivo* and *in vitro* (de Figueiredo, Drecktrah et al. 1998; de Figueiredo, Drecktrah et al. 2000; de Figueiredo, Doody et al. 2001; Brown, Chambers et al. 2003). PLA_2 enzymes increase lysophospholipids which have more positive curvature on the cytosolic leaflet of the membrane; a process which may be necessary for membrane tubules to emanate from organelles. Since PLA_2 enzymes have been linked to membrane tubules in other organelles, PLA_2 enzymes may also have a role in forming membrane tubule transport carriers at the TGN.

Here I used a pharmacological approach to examine the role of PLA_2 enzymes in the formation of membrane tubules at the TGN through the use of specific inhibitors. The TGN tubules formed by PKD-KD were used as a tool to visualize the effects of these PLA_2 antagonists. Here I show that TGN

tubules require PLA_2 enzymes upstream of PKD in the biogenesis of TGN tubules, and that these tubules are important for post-Golgi trafficking.

Methods and Materials

DNA Constructs

PKD-KD-GST and ssHRP-FLAG were both generous gifts from Dr. Vivek Malhotra. PKD-KD was cut from the parent vector with SmaI and SalI and ligated into pEGFP-C1 (Clontech, Mountain View, CA) to generate PKD-KD-GFP. ts045 VSVG-YFP was a generous gift from Dr. Brian Storrie and was cut from the parent vector using BamHI and XhoI and ligated into pEDsRed N-1 (Clontech, Mountain View, CA). All cell culture, ONO treatments, ssHRP assays, and VSV-G assays were done as previously described in Chapters 2, 3, and 4.

Results

PKD-KD-Induced TGN Tubules are inhibited by PLA_2 Antagonists

PLA_2 enzymes have been implicated in multiple membrane trafficking events, specifically those involving the formation of membrane tubules or pleiomorphic membrane transport carriers. The TGN has been shown to form clathrin-coated vesicles, membrane tubules, and vesicular-tubular clusters (VTC) that transport secretory cargo to the plasma membrane and endosomes. I tested if the PLA_2 inhibitor ONO has a role in the formation of these transport carriers. The kinase dead form of PKD (PKD-KD) is known for generating dramatic and abundant TGN tubules, which are thought to result from the impediment of membrane tubule fission. Cells transfected with PKD-

KD-GFP were shown to have numerous TGN membrane tubules, but cells treated with ONO did not (Figure 5-1a). Fewer cells contained TGN tubules as early as 15 min after ONO addition, and by 90 min almost no cells contained tubules (Figure 5-1b).

To test if TGN tubule inhibition is concentration-dependent, various concentrations of ONO were added to cells for 30 min and the number of cells with TGN tubules quantified (Figure 5-2). As the concentration of ONO increased, the number of cells with TGN tubules decreased and plateaued at 20 μ*M*. A second cytoplasmic PLA_2 antagonist, BEL, was also tested for its ability to induce changes in TGN tubules. At 10 μ*M*, BEL effectively inhibited TGN tubules generated by PKD-KD as well (Figure 5-3). Similar to ONO, the number of cells with TGN tubules was quantified and showed that BEL significantly diminished the number of cells with TGN tubules after 15 min.

PLA_2 Activity is Required for the Formation of TGN Tubules

BEL is a suicide substrate and therefore irreversible, but ONO is reversible (de Figueiredo, Drecktrah et al. 1998). Cells transfected with PKD-KD were treated with ONO for 60 min to ensure that nearly all cells lacked TGN tubules. When cells were washed free of ONO, tubules reformed at the TGN within minutes (Figure 5-4). Taken together, these data show that the PLA_2 antagonists ONO and BEL appear to have a direct role in TGN tubules that contain the fission machinery PKD.

Although ONO decreases the number of cells containing PKD-KD TGN tubules, the fate of those pre-existing tubules after addition of ONO is unclear. The tubules may break off from the TGN or retract. Since PKD-KD inhibits

Figure 5-1: The effects of ONO on PKD-KD TGN tubules. HeLa cells transfected with PKD-KD-GFP were incubated with 10 *μM* ONO-Rs-082 or a solvent control for various periods of time. (a) Within 30 min, cells without ONO had abundant TGN tubules containing PKD-KD-GFP while in cells treated with ONO for 30 min, few cells contained tubules. (b) Cells were counted as containing TGN tubules, or not for each time point.

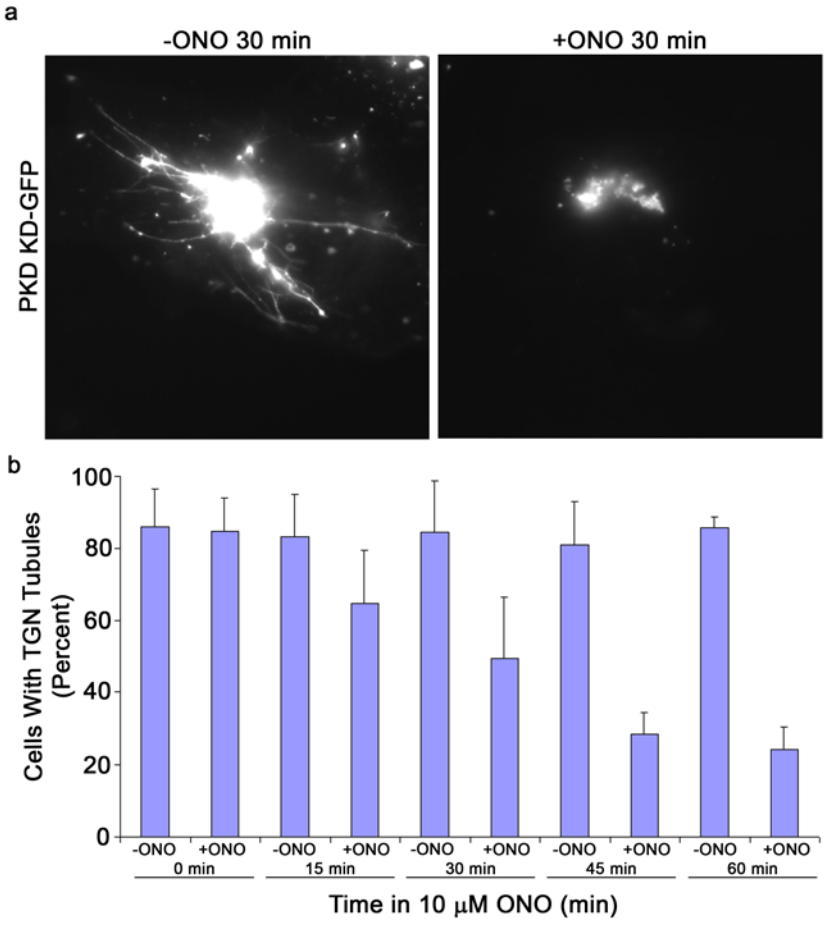
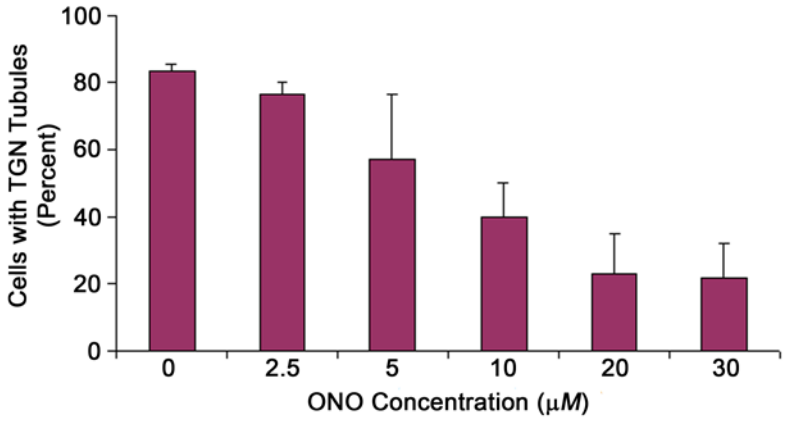

a
-ONO 30 min
+ONO 30 min
PKD KD-GFP
b
Cells With TGN Tubules (Percent)
100
80
60
40
20
0
-ONO
+ONO
0 min
15 min
30 min
45 min
60 min
Time in 10 μM ONO (min)

Figure 5-2: Inhibition of TGN tubule formation is ONO concentration-dependent. HeLa cells transfected with PKD-KD-GFP were treated with 0, 2.5, 5, 10, 20, and 30 *μM* ONO for 30 min. The number of cells with TGN tubules was counted.


Cells with TGN Tubules (Percent)
100
80
60
40
20
0
0
2.5
5
10
20
30
ONO Concentration (μM)

fission, the latter hypothesis is favored. To determine the fate of TGN tubules after the addition of ONO, live cell imaging was used to follow existing tubules. Figure 5-5 shows that the TGN tubules containing PKD-KD are very dynamic and are in a constant state of extension and retraction, as well as breaking away from the TGN. In cells treated with ONO, tubules either break off or retract, but they are not replaced with new tubules. This implies a role for PLA_2 enzymes in the formation, but not necessarily the maintenance or fission, of tubules.

Constitutive TGN-to-Plasma Membrane Trafficking Requires PLA_2 Activity

Although PLA_2 antagonists clearly inhibit TGN membrane tubules generated by PKD-KD, their effect on constitutive export is not known. To see if ONO blocks all TGN transport, a secreted form of horseradish peroxidase (ssHRP) was used to monitor secretion. Cells expressing PKD-KD have been shown previously to slow, although not completely block, the secretion of ssHRP. We found that cells treated with ONO were significantly inhibited in ssHRP secretion, even more so than PKD-KD (Figure 5-6). As expected, cells with both PKD-KD and ONO had no significant secretion. These results indicate an important role for PLA_2 enzymes in generating constitutive TGN transport carriers.

While the transport and secretion of ssHRP is constant throughout the secretory pathway, ONO could inhibit transport of ssHRP at multiple stages along the secretory pathway, not just for the TGN. Therefore, we employed the temperature sensitive vesicular stomatitis virus G protein (ts045 VSV-G) which can be monitored at individual trafficking steps including ER-to-Golgi

Figure 5-3: BEL inhibition of PKD-KD TGN tubules. HeLa cells transfected with PKD-KD-GFP were treated with 10 *μM* BEL (HELSS) for 0, 15, 30, 60, and 90 min. The number of cells containing TGN tubules were counted.

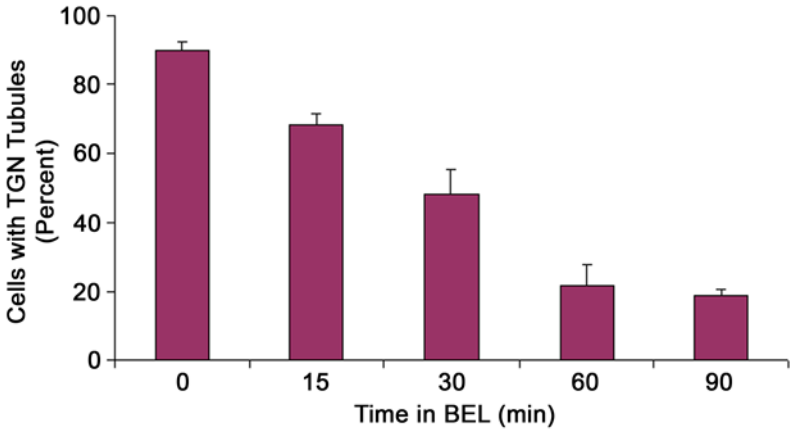

Cells with TGN Tubules (Percent)
100
80
60
40
20
0
0
15
30
60
90
Time in BEL (min)

Figure 5-4: ONO washout allows TGN tubules to reform. HeLa cells transfected with PKD-KD-GFP were treated with 10 *μM* ONO for 60 min. The media was then replaced without ONO. (a) Within 15 min of ONO washout, the TGN reformed tubules which became more numerous and extensive after 30 and 60 min. (b) Cells were counted for containing TGN tubules at various time points after ONO washout. After 60 min, nearly all cells contained tubules.

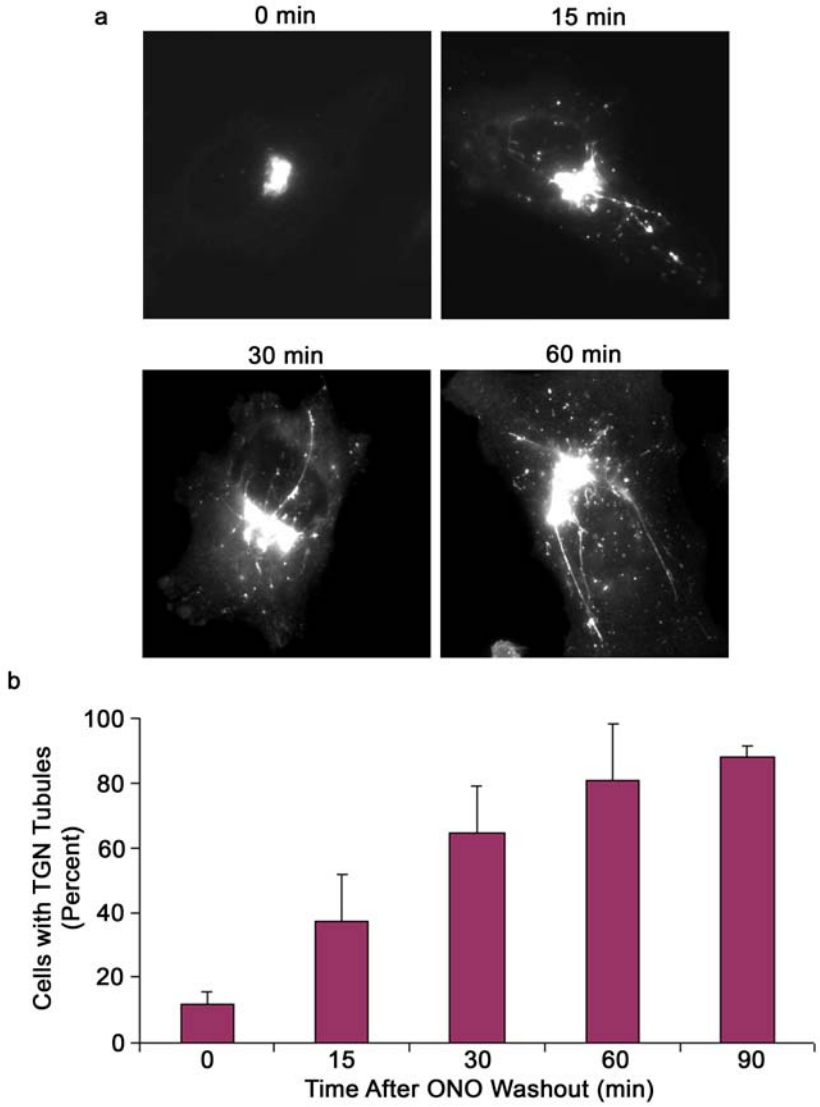
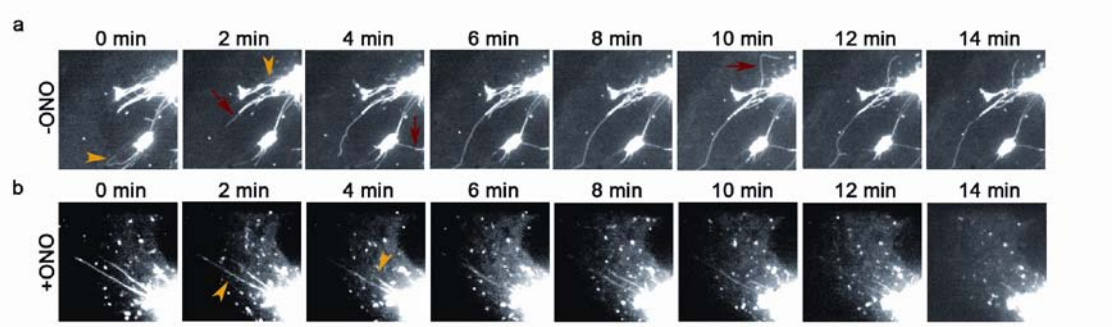

a
0 min
15 min
30 min
60 min
b
Cells with TGN Tubules (Percent)
100
80
60
40
20
0
0
15
30
60
90
Time After ONO Washout (min)

Figure 5-5: Live cell imaging of cells expressing PKD-KD-GFP. (a) In untreated cells, membrane tubules are dynamic in that they form, retract, and break off. (b) In cells treated with 10 *μM* ONO, membrane tubules similarly retract and break off, but do not re-form. Red arrows and yellow triangles indicate, respectively, forming/growing or retracting membrane tubules.


a
0 min
2 min
4 min
6 min
8 min
10 min
12 min
14 min
-ONO
b
0 min
2 min
4 min
6 min
8 min
10 min
12 min
14 min
+ONO

and TGN-to-plasma membrane. I used the 40°C temperature block to accumulate ts045 VSV-G-DsRed in the ER for 18 h, and then I shifted the cells to 20°C for 3 h to accumulate the protein in the TGN. Transfected cells were then shifted to the permissive temperature of 32°C to allow the ts045 VSV-G-DsRed to be transported to the plasma membrane. Both control cells and cells treated with ONO showed an accumulation of VSV-G in the TGN at 20°C. However, 60 min following the shift to 32°C, only control cells efficiently transported VSV-G to the plasma membrane (Figure 5-7). Cells treated with ONO retained VSV-G in the TGN at similar levels seen before the temperature shift. Control cells also contained many TGN tubules with VSV-G, while ONO-treated cells did not have any tubules (not shown). This strongly indicates a role for PLA_2 enzymes in generating transport carriers at the TGN.

Discussion

Membrane trafficking from the TGN to the plasma membrane is a critical step in the secretory pathway. Hirschberg et al showed that many proteins, including VSV-G, are transported out of the TGN in membrane tubules that break off from the donor organelle (Hirschberg, Miller et al. 1998). These tubules are particularly suited for large proteins such as collagen that cannot fit into small 60 nm coated vesicles. Recently, both PKD and BARS-50 have been implicated in the fission of these tubules. However, very little in known about how the tubules are formed (Bard and Malhotra 2006).

Previous studies have indicated that PLA_2 enzymes are important for membrane tubule biogenesis. PLA_2 enzymes are important for BFA-stimulated Golgi membrane tubule formation as well as constitutive retrograde Golgi tubule formation (de Figueiredo, Drecktrah et al. 2000). PLA_2 enzymes

Figure 5-6: ONO blocks TGN trafficking of ssHRP. HeLa cells were transfected with empty vector or PKD-KD-GFP for 24 hours followed by transfection with ssHRP-FLAG for 24 hours. Cells media was replaced and media samples were taken at 2, 4, 6, and 8 hours. Cells treated with 10 *μM* ONO had complete inhibition of ssHRP secretion and cells overexpressing PKD-KD-GFP had approximately 50% reduction in secretion.

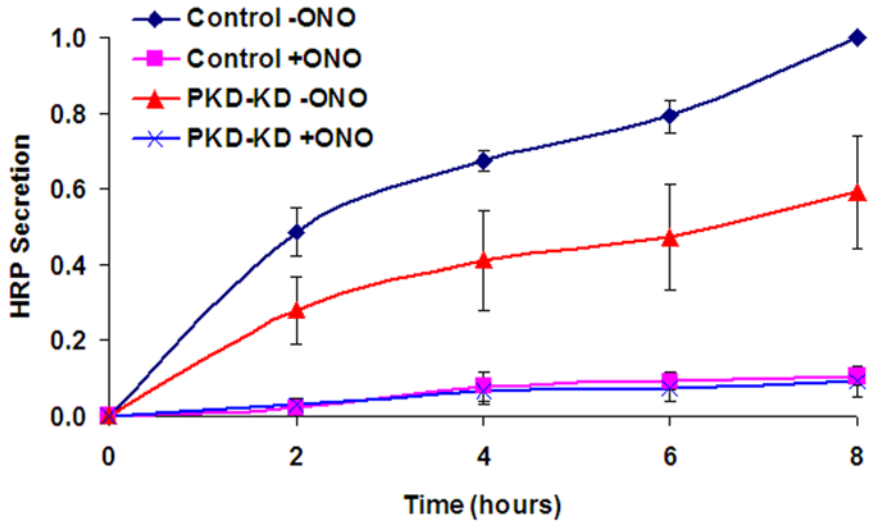
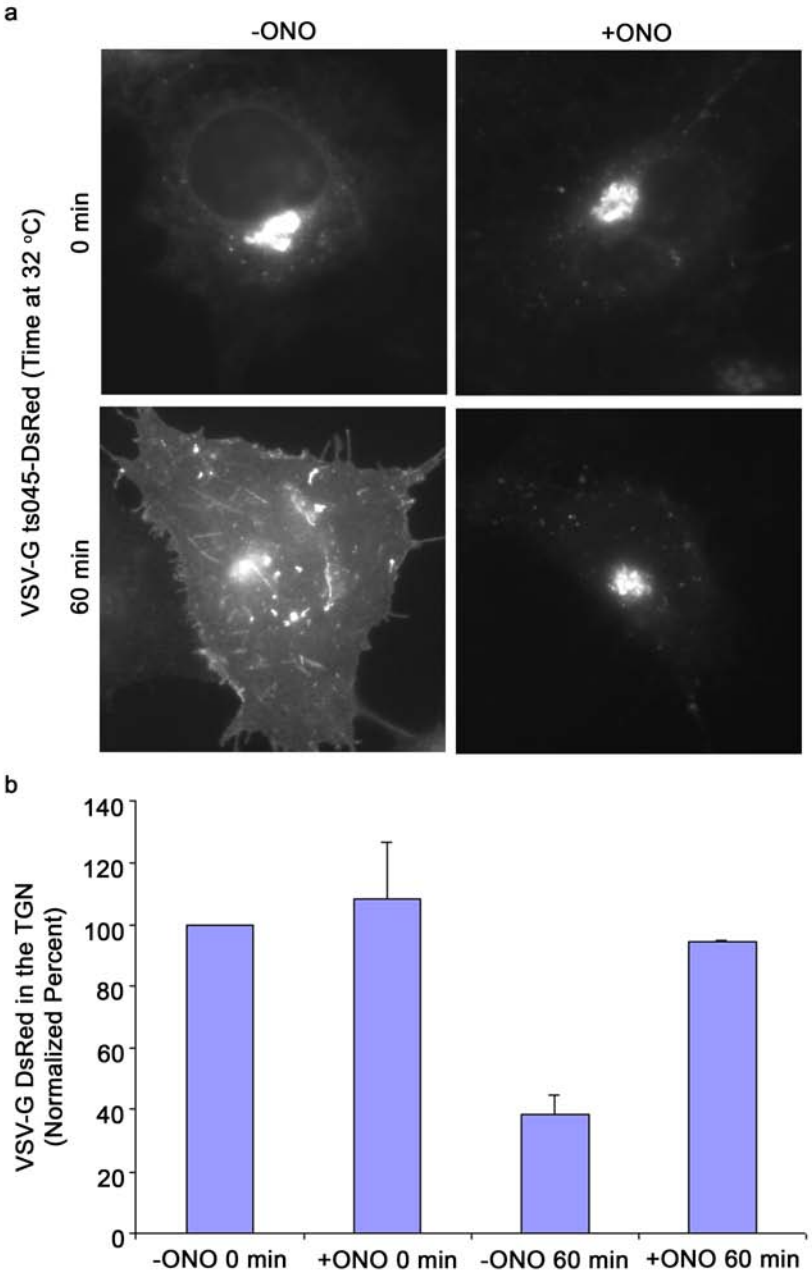

Control -ONO
Control +ONO
PKD-KD -ONO
PKD-KD +ONO
HRP Secretion
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
Time (hours)

Figure 5-7: ONO block TGN trafficking of VSV-G ts045-DsRed. Cells transfected with Ts045 VSV-G-DsRed were incubated at 40 °C for 18 hours then 20 °C for 3 hours and then permissive temperature of 32 °C. (a) Cells without ONO readily transported VSV-G to the plasma membrane within 60 min at permissive temperature while cells treated with 10 *μM* ONO retained VSV-G in the TGN. (b) TGN-localized VSV-G was quantified by measuring fluorescence intensity of DsRed in the TGN versus the entire cell. Numbers were normalized to control and demonstrate that ONO blocked Ts045 VSV-G-DsRed escape from the TGN.


a
-ONO
+ONO
VSV-G ts045-DsRed (Time at 32 °C)
0 min
60 min
b
VSV-G DsRed in the TGN
(Normalized Percent)
140
120
100
80
60
40
20
0
-ONO 0 min
+ONO 0 min
-ONO 60 min
+ONO 60 min

are also critical for generating tubular bridges that connect Golgi stacks (Brown, Chambers et al. 2003). This study adds to our understanding of PLA_2 enzymes in trafficking.

Here I show that the PLA_2 antagonist ONO can inhibit TGN tubules induced by the overexpression of PKD-KD. The effect is concentration-dependent as well as reversible. In addition, ONO also blocks TGN to plasma membrane transport of both soluble cargo (ssHRP) and transmembrane cargo (VSV-G).

Using live cell imaging, I observed the dynamics of TGN tubules generated by PKD-KD for the first time. These tubules are very dynamic and constantly grow and retract. I believe that ONO has little effect on existing tubules, but prevents the formation of new tubules. This is supported by the fact that washing out ONO caused the rapid formation of new TGN tubules; moreover, VSV-G was never seen in tubules when cells were treated with ONO, but VSV-G containing tubules were plentiful in control cells.

A second PLA_2 antagonist, BEL, also inhibited TGN tubules. ONO is a $cPLA_2$ inhibitor with an IC_{50} of 10 μ*M*, however, BEL inhibits Ca^{2+}-independent-PLA_2 enzymes ($iPLA_2$ enzymes) at 1 μ*M* and Ca^{2+}-dependent-PLA_2 enzymes ($cPLA_2$ enzymes) at 10 μ*M* (de Figueiredo, Drecktrah et al. 1998). Little effect was seen when BEL was used at 1 μ*M* (not shown), but membrane tubules were inhibited at 10 μ*M*. This implies that a $cPLA_2$ may be responsible for the formation of TGN tubules. Evidence now exists for both $iPLA_2$ enzymes and $cPLA_2$ enzymes in membrane trafficking.

Here I have shown that PLA_2 activity is critical for export from the TGN. We hypothesize that PLA_2 enzymes generate lysophospholipids (LPLs) with large head groups and a fatty acid. An accumulation of LPLs may be able to

alter the membrane from a flat surface to a curved membrane, since LPLs are shaped like inverted cones, while cylindrical phospholipids do not. This change in membrane morphology may be critical for forming the shape of a membrane tubule, or the binding of proteins. Phospholipids appear to have key roles not only in the scission of transport carriers, but also in their formation.

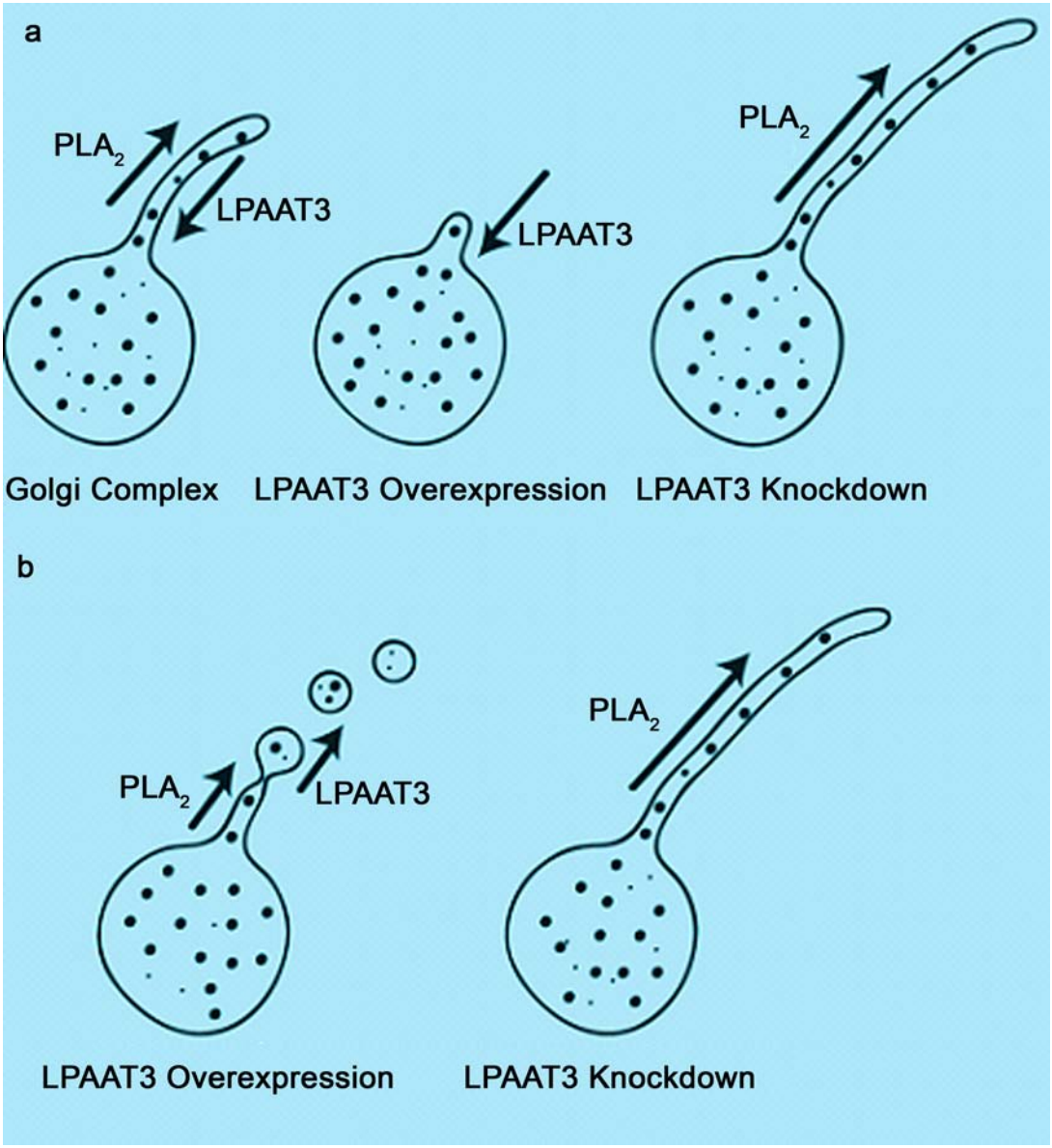
CHAPTER 6

Conclusions

The role of LPAAT activity in membrane trafficking was first suspected through the use of the LPAAT antagonist CI-976. This inhibitor was an extremely useful tool for looking at the role of LPAATs in the formation of membrane tubules and vesicles and how LPAATs impact specific trafficking pathways. One shortcoming was that no specific LPAAT had been identified as a potential target of CI-976. Since the CI-976-sensitive LPAAT activity was tightly associated with the Golgi membranes, and could not be stripped away by high concentrations of salt, transmembrane LPAATs were explored as potential CI-976 targets (Chambers and Brown 2004). LPAAT3 is one of many transmembrane LPAATs identified by sequence alone, and was found to be localized to Golgi and ER membranes.

I performed a biochemical analysis of LPAAT3 which showed that LPAAT3 used lysophosphatidic acid (LPA) and acyl-CoA substrates to make phosphatidic acid (PA). This activity is located in the Golgi membranes themselves indicating that overexpression of LPAAT3 can increase PA levels in the membrane. This conversion between LPA and PA can alter membrane curvature, which is important for membrane trafficking and organelle structure. LPAAT3 indeed has an effect on Golgi membrane structure. I found that overexpression of LPAAT3 appears to preserve the compact structure against fragmenting agents such as ONO. Conversely, when I knocked down LPAAT3, the Golgi fragmented into individual mini-stacks that could be seen using electron microscopy. This implies that phospholipid metabolism has a

Figure 6-1: Model for PLA_2s and LPAATs in forming tubules. (a) PLA_2s and LPAATs may have antagonistic roles where PLA_2s form membrane tubules and LPAATs retract tubules. (b) Alternatively, PLA_2s may form membrane tubules which are then consumed by LPAATs in a more cooperative mechanism.

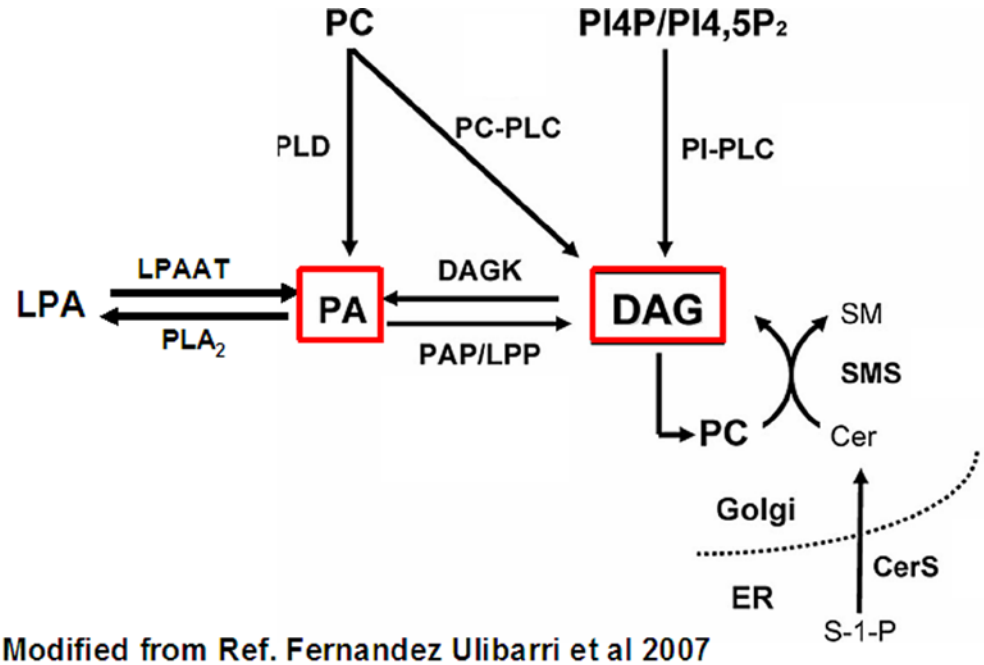

a
PLA2
LPAAT3
LPAAT3
PLA2
Golgi Complex
LPAAT3 Overexpression
LPAAT3 Knockdown
b
PLA2
LPAAT3
PLA2
LPAAT3 Overexpression
LPAAT3 Knockdown

role in maintaining Golgi complex structure. I investigated the structure of LPAAT3 and generated a model for LPAAT3 topology, which places critical domains on both the cytoplasmic and luminal sides of the membrane. The exact purpose of this is unclear, but both cytoplasmic and luminal loops are important for LPAAT catalytic activity. My topology model for LPAAT3 is similar to one recently reported for LPAAT1 (Yamashita, Nakanishi et al. 2007).

Next, I examined LPAAT3 for a potential role in membrane trafficking. Overexpression of LPAAT3 and knockdown of LPAAT3 proved to be useful tools in understanding the role of LPAATs and phospholipids in Golgi trafficking. Cells overexpressing LPAAT3 were much slower to form Golgi tubules when stimulated with either BFA or CI-976. LPAAT3 also slowed the Golgi to ER retrograde trafficking of endogenous ERGIC-53/p58. A catalytically dead mutant of LPAAT3, however, had no effect. These results support previous observations that inhibition of LPAATs can promote tubules. LPAAT3 knockdown accelerated Golgi tubule formation and retrograde trafficking. While neither overexpression nor knockdown of LPAAT3 caused a complete defect in Golgi trafficking, phospholipid metabolism is redundant and when one pathway is altered, a cell can compensate by using less efficient alternative pathways.

LPAAT3 overexpression similarly had an effect on anterograde trafficking from the Golgi to the plasma membrane. I tested the secretion of a small protein ssHRP. In cells overexpressing LPAAT3, ssHRP secretion was slower and in cells knocked down for LPAAT3, it was faster. Cells overexpreesing LPAAT3 mutant had similar secretion kinetics as control cells. The rapid secretion observed in cells knocked down for LPAAT3 could be

Figure 6-2: Lipid Metabolic Pathways. Phospholipid metabolic pathways that may have important roles in membrane trafficking including phosphatidic acid (PA), diacylglycerol (DAG) and ceremides. Figure is modified from (Fernandez-Ulibarri, Vilella et al. 2007).

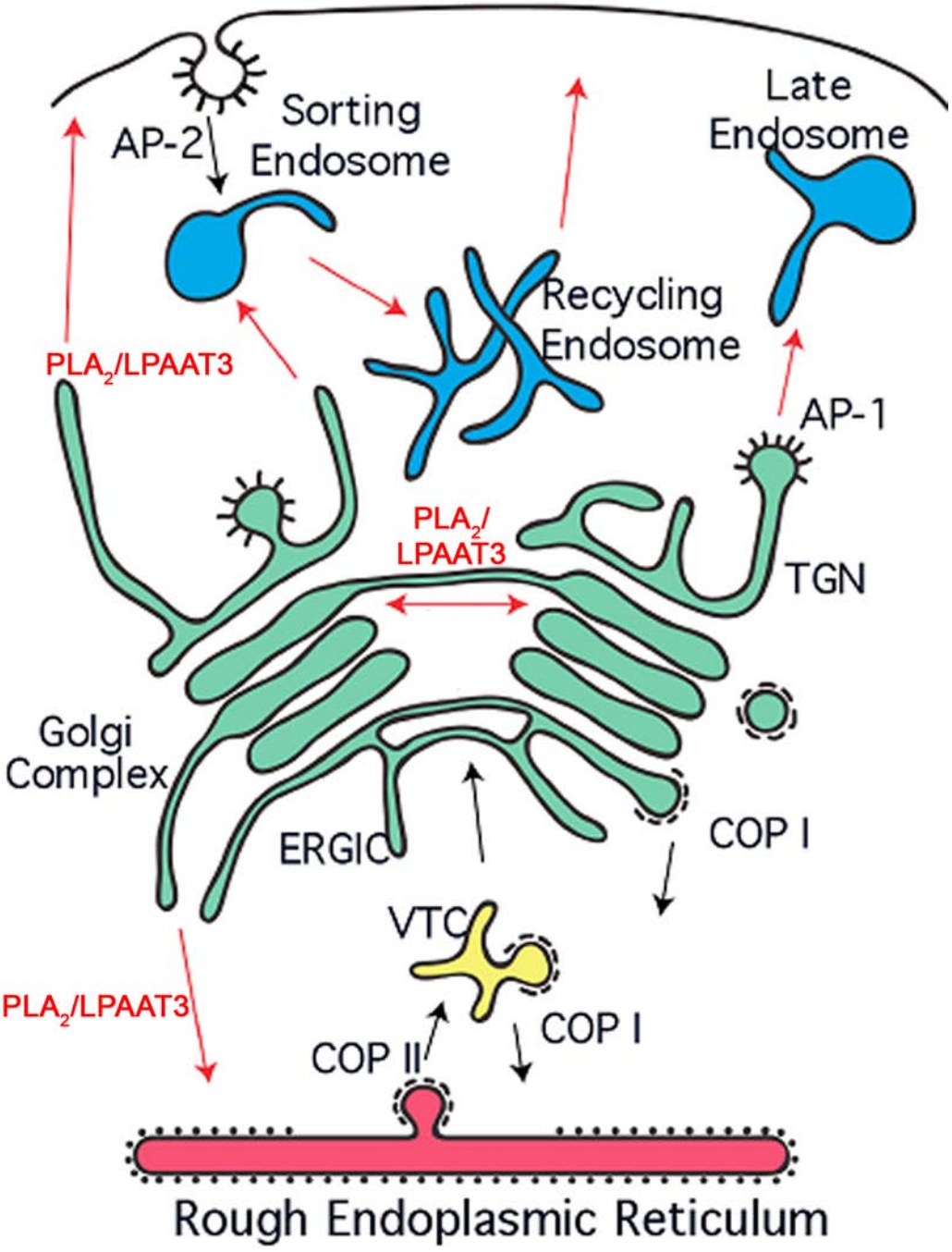


Modified from Ref. Fernandez Ulibarri et al 2007

slowed by the expression of a siRNA-resistant version of LPAAT3. The transmembrane protein VSV-G was also slower to arrive at the plasma membrane and accumulated in the Golgi complex for both LPAAT3 overexpression and knockdown cells. It is unclear why LPAAT3 knockdown would slow VSV-G trafficking, but measurements may have been misleading since the Golgi is fragmented in those cells, which made measuring Golgi fluorescence very difficult. Nevertheless, LPAAT3 has an effect on Golgi trafficking including retrograde trafficking from the Golgi to the ER and anterograde trafficking from the Golgi to the cell surface.

LPAAT3 may alter Golgi trafficking by regulating membrane tubules. Membrane tubules are involved in constitutive retrograde trafficking and have been observed forming transport carriers at the TGN. Alternatively, LPAAT3 may have a role in forming Golgi vesicles for the transport of cargo molecules. Figure 6-1 shows these two models for how LPAATs may affect membrane tubules. To test these hypotheses, I assayed for Golgi membrane tubule formation *in vitro* in cells overexpressing LPAAT3. The isolated Golgi failed to form tubules like the control Golgi. In cells treated with BFA, the number and lengths of Golgi tubules were measured in cells overexpressing LPAAT3. I found that cells overexpressing LPAAT3 formed fewer membrane tubules, shorter membrane tubules, and tubules that took longer to form. Finally, *in vitro* COPI vesicle budding assays showed that while LPAAT3 Golgi formed slightly fewer vesicles; however, it was not statistically significant. This has led us to the conclusion that LPAAT3 may be a negative regulator of membrane tubules, and that LPAATs may consume lysophospholipids generated by PLA_2 enzymes (Figure 6-1a).

Figure 6-3: Secretory and retrograde trafficking pathways dependant on PLA_2 and LPAAT activity. This study has demonstrated the roles of LPAAT3 and PLA_2s in various membrane trafficking pathways including anterograde and retrograde pathways. This may be due to their abilities to form/regulate membrane tubules. Pathways that involve the activity of PLA_2s and LPAAT3 in this study are labeled in red.


AP-2
Sorting Endosome
Late Endosome
Recycling Endosome
PLA2/LPAAT3
AP-1
PLA2/ LPAAT3
TGN
Golgi Complex
ERGIC
COP I
VTC
PLA2/LPAAT3
COP II
COP I
Rough Endoplasmic Reticulum

PLA_2 enzymes may generate lysophospholipids to form membrane tubules and LPAATs may reduce lysophospholipids. While a great deal is known about PLA_2 enzymes and retrograde trafficking, less is known about PLA_2 enzymes in TGN trafficking. However, membrane tubules are important for generating TGN transport carriers. The PLA_2 antagonists ONO and BEL were used to look at TGN tubules formed by the protein PKD-KD (protein kinase D – kinase dead). PLA_2 antagonists appear to be required for these tubules to form in a concentration-dependent manner. ONO also inhibited TGN trafficking including trafficking of the aforementioned ssHRP and VSV-G. This adds to our understanding of how PLA_2 enzymes are important for membrane tubule formation and TGN trafficking.

Lipid turnover is the iterative synthesis and hydrolysis of phospholipids, neutral lipids, sphingolipids, and ceramides within the cell. Numerous biosynthetic and metabolic pathways link different lipid species allowing a cell to rapidly alter lipid content without having to make new lipids from basic components. LPAAT3 can regulate levels of LPA and PA within membranes. Overexpression of LPAAT3 increases PA levels and decreases LPA levels while LPAAT3 knockdown does the reverse. LPA and PA may have direct influences on membrane curvature, cell signaling, or protein recruitment; however, changes in LPA and PA may have an indirect effect on cellular levels of DAG or other phospholipids. These downstream products may also have effects on trafficking; therefore LPAAT3 expression might have unforeseen effects on lipid turnover. Treatment of cells with the PLA_2 inhibitor ONO may also affect lipid turnover. ONO increases phospholipids and decreases lysophospholipids. It is still unclear which phospholipids are altered by ONO. Likewise, other downstream phospholipid metabolites may also be changed by

cells treated with ONO. Some of the pathways involved in lipid turnover are shown in Figure 6-2, which has been modified from Fernandez-Ulibarri et al (Fernandez-Ulibarri, Vilella et al. 2007).

LPAAT3 is the first LPAAT to be localized to the Golgi complex and the first LPAAT shown to have a role in membrane trafficking. We have a clearer understanding of how LPAATs and PLA_2 enzymes are involved in altering phospholipids in organelle membranes and how those changes can affect critical cellular functions like membrane trafficking. Key trafficking pathways that use PLA_2 enzymes and LPAAT3 are shown in Figure 6-3. Although some questions still remain about which phospholipid species are the predominant players in trafficking and what pathways are the primary metabolic routes to synthesizing and regulating those lipids, it is apparent that phospholipids are as important as their protein counterparts in secretion.

REFERENCES

Agarwal, A. K., R. I. Barnes, et al. (2006). "Functional characterization of human 1-acylglycerol-3-phosphate acyltransferase isoform 8: cloning, tissue distribution, gene structure, and enzymatic activity." Arch Biochem Biophys **449**(1-2): 64-76.

Agarwal, A. K., S. Sukumaran, et al. (2007). "Functional characterization of human 1-acylglycerol-3-phosphate-O-acyltransferase isoform 9: cloning, tissue distribution, gene structure, and enzymatic activity." J Endocrinol **193**(3): 445-57.

Anderson, R. G., J. R. Falck, et al. (1984). "Visualization of acidic organelles in intact cells by electron microscopy." Proc Natl Acad Sci U S A **81**(15): 4838-42.

Aridor, M., J. Weissman, et al. (1998). "Cargo selection by the COPII budding machinery during export from the ER." J Cell Biol **141**(1): 61-70.

Arstikaitis, P. and C. Gauthier-Campbell (2006). "BARS at the synapse: PICK-1 lipid binding domain regulates targeting, trafficking, and synaptic plasticity." J Neurosci **26**(26): 6909-10.

Asp, L., F. Kartberg, et al. (2009). "Early stages of Golgi vesicle and tubule formation require diacylglycerol." Mol Biol Cell **20**(3): 780-90.

Balsinde, J., M. V. Winstead, et al. (2002). "Phospholipase A(2) regulation of arachidonic acid mobilization." FEBS Lett **531**(1): 2-6.

Bannykh, S. I., T. Rowe, et al. (1996). "The organization of endoplasmic reticulum export complexes." J Cell Biol **135**(1): 19-35.

Banta, M., R. S. Polizotto, et al. (1995). "Characterization of a cytosolic activity that induces the formation of Golgi membrane tubules in a cell-free reconstitution system." Biochemistry **34**(41): 13359-66.

Bard, F. and V. Malhotra (2006). "The formation of TGN-to-plasma-membrane transport carriers." Annu Rev Cell Dev Biol **22**: 439-55.

Barlowe, C., L. Orci, et al. (1994). "COPII: a membrane coat formed by Sec proteins that drive vesicle budding from the endoplasmic reticulum." Cell **77**(6): 895-907.

Baron, C. L. and V. Malhotra (2002). "Role of diacylglycerol in PKD recruitment to the TGN and protein transport to the plasma membrane." Science **295**(5553): 325-8.

Beigneux, A. P., L. Vergnes, et al. (2006). "Agpat6--a novel lipid biosynthetic gene required for triacylglycerol production in mammary epithelium." J Lipid Res **47**(4): 734-44.

Bielli, A., C. J. Haney, et al. (2005). "Regulation of Sar1 NH2 terminus by GTP binding and hydrolysis promotes membrane deformation to control COPII vesicle fission." J Cell Biol **171**(6): 919-24.

Blobel, G. (1987). "How proteins move across the endoplasmic reticulum membrane." Hepatology **7**(1 Suppl): 26S-29S.

Blobel, G. and B. Dobberstein (1975). "Transfer of proteins across membranes. I. Presence of proteolytically processed and unprocessed nascent immunoglobulin light chains on membrane-bound ribosomes of murine myeloma." J Cell Biol **67**(3): 835-51.

Blobel, G. and B. Dobberstein (1975). "Transfer of proteins across membranes. II. Reconstitution of functional rough microsomes from heterologous components." J Cell Biol **67**(3): 852-62.

Bonazzi, M., S. Spano, et al. (2005). "CtBP3/BARS drives membrane fission in dynamin-independent transport pathways." Nat Cell Biol **7**(6): 570-80.

Bonifacino, J. S. and B. S. Glick (2004). "The mechanisms of vesicle budding and fusion." Cell **116**(2): 153-66.

Bonifacino, J. S. and R. Rojas (2006). "Retrograde transport from endosomes to the trans-Golgi network." Nat Rev Mol Cell Biol **7**(8): 568-79.

Bretscher, M. S. and S. Munro (1993). "Cholesterol and the Golgi apparatus." Science **261**(5126): 1280-1.

Brown, W. J., K. Chambers, et al. (2003). "Phospholipase A2 (PLA2) enzymes in membrane trafficking: mediators of membrane shape and function." Traffic **4**(4): 214-21.

Brown, W. J., H. Plutner, et al. (2008). "The lysophospholipid acyltransferase antagonist CI-976 inhibits a late step in COPII vesicle budding." Traffic **9**(5): 786-97.

Brown, W. J. and J. A. Schmidt (2005). "Use of acyltransferase inhibitors to block vesicular traffic between the ER and Golgi complex." Methods Enzymol **404**: 115-25.

Burkhardt, J. K., C. J. Echeverri, et al. (1997). "Overexpression of the dynamitin (p50) subunit of the dynactin complex disrupts dynein-dependent maintenance of membrane organelle distribution." J Cell Biol **139**(2): 469-84.

Chambers, K. and W. J. Brown (2004). "Characterization of a novel CI-976-sensitive lysophospholipid acyltransferase that is associated with the Golgi complex." Biochem Biophys Res Commun **313**(3): 681-6.

Chambers, K., B. Judson, et al. (2005). "A unique lysophospholipid acyltransferase (LPAT) antagonist, CI-976, affects secretory and endocytic membrane trafficking pathways." J Cell Sci **118**(Pt 14): 3061-71.

Chen, Y. Q., M. S. Kuo, et al. (2008). "AGPAT6 is a novel microsomal glycerol-3-phosphate acyltransferase." J Biol Chem **283**(15): 10048-57.

Chernomordik, L. V. and M. M. Kozlov (2005). "Membrane hemifusion: crossing a chasm in two leaps." Cell **123**(3): 375-82.

Cluett, E. B., S. A. Wood, et al. (1993). "Tubulation of Golgi membranes in vivo and in vitro in the absence of brefeldin A." J Cell Biol **120**(1): 15-24.

Cole, N. B., J. Ellenberg, et al. (1998). "Retrograde transport of Golgi-localized proteins to the ER." J Cell Biol **140**(1): 1-15.

Coleman, J. (1990). "Characterization of Escherichia coli cells deficient in 1-acyl-sn-glycerol-3- phosphate acyltransferase activity." J Biol Chem **265**(28): 17215-21.

Coleman, J. (1992). "Characterization of the Escherichia coli gene for 1-acyl-sn-glycerol-3-phosphate acyltransferase (plsC)." Mol Gen Genet **232**(2): 295-303.

Dabora, S. L. and M. P. Sheetz (1988). "The microtubule-dependent formation of a tubulovesicular network with characteristics of the ER from cultured cell extracts." Cell **54**(1): 27-35.

Dahms, N. M., P. Lobel, et al. (1989). "Mannose 6-phosphate receptors and lysosomal enzyme targeting." J Biol Chem **264**(21): 12115-8.

Dalton, A. J. and M. D. Felix (1954). "Cytologic and cytochemical characteristics of the Golgi substance of epithelial cells of the epididymis in situ, in homogenates and after isolation." Am J Anat **94**(2): 171-207.

Dalton, A. J. and M. D. Felix (1956). "A comparative study of the Golgi complex." J Biophys Biochem Cytol **2**(4, Suppl): 79-84.

de Figueiredo, P., A. Doody, et al. (2001). "Inhibition of transferrin recycling and endosome tubulation by phospholipase A2 antagonists." J Biol Chem **276**(50): 47361-70.

de Figueiredo, P., D. Drecktrah, et al. (1998). "Evidence that phospholipase A2 activity is required for Golgi complex and trans Golgi network membrane tubulation." Proc Natl Acad Sci U S A **95**(15): 8642-7.

de Figueiredo, P., D. Drecktrah, et al. (2000). "Phospholipase A2 antagonists inhibit constitutive retrograde membrane traffic to the endoplasmic reticulum." Traffic **1**(6): 504-11.

de Figueiredo, P., R. S. Polizotto, et al. (1999). "Membrane tubule-mediated reassembly and maintenance of the Golgi complex is disrupted by phospholipase A2 antagonists." Mol Biol Cell **10**(6): 1763-82.

Deshaies, R. J., S. L. Sanders, et al. (1991). "Assembly of yeast Sec proteins involved in translocation into the endoplasmic reticulum into a membrane-bound multisubunit complex." Nature **349**(6312): 806-8.

Diaz Anel, A. M. and V. Malhotra (2005). "PKCeta is required for beta1gamma2/beta3gamma2- and PKD-mediated transport to the cell surface and the organization of the Golgi apparatus." J Cell Biol **169**(1): 83-91.

Donaldson, J. G., D. Finazzi, et al. (1992). "Brefeldin A inhibits Golgi membrane-catalysed exchange of guanine nucleotide onto ARF protein." Nature **360**(6402): 350-2.

Drecktrah, D. and W. J. Brown (1999). "Phospholipase A(2) antagonists inhibit nocodazole-induced Golgi ministack formation: evidence of an ER intermediate and constitutive cycling." Mol Biol Cell **10**(12): 4021-32.

Drecktrah, D., K. Chambers, et al. (2003). "Inhibition of a Golgi complex lysophospholipid acyltransferase induces membrane tubule formation and retrograde trafficking." Mol Biol Cell **14**(8): 3459-69.

Eberhardt, C., P. W. Gray, et al. (1997). "Human lysophosphatidic acid acyltransferase. cDNA cloning, expression, and localization to chromosome 9q34.3." J Biol Chem **272**(32): 20299-305.

Farquhar, M. G. and G. E. Palade (1998). "The Golgi apparatus: 100 years of progress and controversy." Trends Cell Biol **8**(1): 2-10.

Fernandez-Ulibarri, I., M. Vilella, et al. (2007). "Diacylglycerol is required for the formation of COPI vesicles in the Golgi-to-ER transport pathway." Mol Biol Cell **18**(9): 3250-63.

Gallop, J. L., P. J. Butler, et al. (2005). "Endophilin and CtBP/BARS are not acyl transferases in endocytosis or Golgi fission." Nature **438**(7068): 675-8.

Glick, B. S. and J. E. Rothman (1987). "Possible role for fatty acyl-coenzyme A in intracellular protein transport." Nature **326**(6110): 309-12.

Golgi, C. (1989). "On the structure of the nerve cells of the spinal ganglia. 1898." J Microsc **155**(Pt 1): 9-14.

Gurkan, C., S. M. Stagg, et al. (2006). "The COPII cage: unifying principles of vesicle coat assembly." Nat Rev Mol Cell Biol **7**(10): 727-38.

Haque, W., A. Garg, et al. (2005). "Enzymatic activity of naturally occurring 1-acylglycerol-3-phosphate-O-acyltransferase 2 mutants associated with congenital generalized lipodystrophy." Biochem Biophys Res Commun **327**(2): 446-53.

Hart, G. W. (1992). "Glycosylation." Curr Opin Cell Biol **4**(6): 1017-23.

Harter, C. and C. Reinhard (2000). "The secretory pathway from history to the state of the art." Subcell Biochem **34**: 1-38.

Hauri, H. P., F. Kappeler, et al. (2000). "ERGIC-53 and traffic in the secretory pathway." J Cell Sci **113 (Pt 4)**: 587-96.

Hirsch, J. G. and M. E. Fedorko (1968). "Ultrastructure of human leukocytes after simultaneous fixation with glutaraldehyde and osmium tetroxide and "postfixation" in uranyl acetate." J Cell Biol **38**(3): 615-27.

Hirschberg, K., C. M. Miller, et al. (1998). "Kinetic analysis of secretory protein traffic and characterization of golgi to plasma membrane transport intermediates in living cells." J Cell Biol **143**(6): 1485-503.

Hirst, J. and M. S. Robinson (1998). "Clathrin and adaptors." Biochim Biophys Acta **1404**(1-2): 173-93.

Itin, C., R. Schindler, et al. (1995). "Targeting of protein ERGIC-53 to the ER/ERGIC/cis-Golgi recycling pathway." J Cell Biol **131**(1): 57-67.

Kaiser, C. A. and R. Schekman (1990). "Distinct sets of SEC genes govern transport vesicle formation and fusion early in the secretory pathway." Cell **61**(4): 723-33.

Klumperman, J., A. Schweizer, et al. (1998). "The recycling pathway of protein ERGIC-53 and dynamics of the ER-Golgi intermediate compartment." J Cell Sci **111 (Pt 22)**: 3411-25.

Kooijman, E. E., V. Chupin, et al. (2005). "Spontaneous curvature of phosphatidic acid and lysophosphatidic acid." Biochemistry **44**(6): 2097-102.

Kreis, T. E. (1990). "Role of microtubules in the organisation of the Golgi apparatus." Cell Motil Cytoskeleton **15**(2): 67-70.

Lands, W. E. (1958). "Metabolism of glycerolipides; a comparison of lecithin and triglyceride synthesis." J Biol Chem **231**(2): 883-8.

Leung, D. W. (2001). "The structure and functions of human lysophosphatidic acid acyltransferases." Front Biosci **6**: D944-53.

Lewin, T. M., P. Wang, et al. (1999). "Analysis of amino acid motifs diagnostic for the sn-glycerol-3-phosphate acyltransferase reaction." Biochemistry **38**(18): 5764-71.

Li, D., L. Yu, et al. (2003). "Cloning and identification of the human LPAAT-zeta gene, a novel member of the lysophosphatidic acid acyltransferase family." J Hum Genet **48**(8): 438-42.

Liljedahl, M., Y. Maeda, et al. (2001). "Protein kinase D regulates the fission of cell surface destined transport carriers from the trans-Golgi network." Cell **104**(3): 409-20.

Lippincott-Schwartz, J., L. C. Yuan, et al. (1989). "Rapid redistribution of Golgi proteins into the ER in cells treated with brefeldin A: evidence for membrane cycling from Golgi to ER." Cell **56**(5): 801-13.

Lorenz, H., D. W. Hailey, et al. (2006). "Fluorescence protease protection of GFP chimeras to reveal protein topology and subcellular localization." Nat Methods **3**(3): 205-10.

Lorenz, H., D. W. Hailey, et al. (2006). "The fluorescence protease protection (FPP) assay to determine protein localization and membrane topology." Nat Protoc **1**(1): 276-9.

Losev, E., C. A. Reinke, et al. (2006). "Golgi maturation visualized in living yeast." Nature **441**(7096): 1002-6.

Lu, B., Y. J. Jiang, et al. (2005). "Cloning and characterization of murine 1-acyl-sn-glycerol 3-phosphate acyltransferases and their regulation by PPARalpha in murine heart." Biochem J **385**(Pt 2): 469-77.

Maeda, Y., G. V. Beznoussenko, et al. (2001). "Recruitment of protein kinase D to the trans-Golgi network via the first cysteine-rich domain." Embo J **20**(21): 5982-90.

Martinez-Menarguez, J. A., R. Prekeris, et al. (2001). "Peri-Golgi vesicles contain retrograde but not anterograde proteins consistent with the cisternal progression model of intra-Golgi transport." J Cell Biol **155**(7): 1213-24.

Matsuura-Tokita, K., M. Takeuchi, et al. (2006). "Live imaging of yeast Golgi cisternal maturation." Nature **441**(7096): 1007-10.

McMahon, H. T. and J. L. Gallop (2005). "Membrane curvature and mechanisms of dynamic cell membrane remodelling." Nature **438**(7068): 590-6.

Mironov, A. A., A. A. Mironov, Jr., et al. (2003). "ER-to-Golgi carriers arise through direct en bloc protrusion and multistage maturation of specialized ER exit domains." Dev Cell **5**(4): 583-94.

Modregger, J., A. A. Schmidt, et al. (2003). "Characterization of Endophilin B1b, a brain-specific membrane-associated lysophosphatidic acid acyl transferase with properties distinct from endophilin A1." J Biol Chem **278**(6): 4160-7.

Morand, L. Z., S. Patil, et al. (1998). "Alteration of the fatty acid substrate specificity of lysophosphatidate acyltransferase by site-directed mutagenesis." Biochem Biophys Res Commun **244**(1): 79-84.

Munro, S. and H. R. Pelham (1987). "A C-terminal signal prevents secretion of luminal ER proteins." Cell **48**(5): 899-907.

Murakami, M. and I. Kudo (2002). "Phospholipase A2." J Biochem **131**(3): 285-92.

Novick, P., C. Field, et al. (1980). "Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway." Cell **21**(1): 205-15.

Orci, L., M. Amherdt, et al. (2000). "Exclusion of golgi residents from transport vesicles budding from Golgi cisternae in intact cells." J Cell Biol **150**(6): 1263-70.

Orci, L., M. Tagaya, et al. (1991). "Brefeldin A, a drug that blocks secretion, prevents the assembly of non-clathrin-coated buds on Golgi cisternae." Cell 64(6): 1183-95.

Palade, G. (1975). "Intracellular Aspects of the Process of Protein Synthesis." Science 189(4206): 867.

Pelham, H. R. (2006). "Maturation of Golgi cisternae directly observed." Trends Biochem Sci 31(11): 601-4.

Perinetti, G., T. Muller, et al. (2009). "Correlation of 4Pi- and electron microscopy to study transport through single Golgi stacks in living cells with super resolution." Traffic.

Pfanner, N., L. Orci, et al. (1989). "Fatty acyl-coenzyme A is required for budding of transport vesicles from Golgi cisternae." Cell 59(1): 95-102.

Polishchuk, E. V., A. Di Pentima, et al. (2003). "Mechanism of constitutive export from the golgi: bulk flow via the formation, protrusion, and en bloc cleavage of large trans-golgi network tubular domains." Mol Biol Cell 14(11): 4470-85.

Presley, J. F., N. B. Cole, et al. (1997). "ER-to-Golgi transport visualized in living cells." Nature 389(6646): 81-5.

Presley, J. F., C. Smith, et al. (1998). "Golgi membrane dynamics." Mol Biol Cell 9(7): 1617-26.

Rapoport, T. A. (2009). "Membrane Shape." Annu Rev Cell Dev Biol.

Sannerud, R., J. Saraste, et al. (2003). "Retrograde traffic in the biosynthetic-secretory route: pathways and machinery." Curr Opin Cell Biol 15(4): 438-45.

Schmidt, A., M. Wolde, et al. (1999). "Endophilin I mediates synaptic vesicle formation by transfer of arachidonate to lysophosphatidic acid." Nature 401(6749): 133-41.

Sciaky, N., J. Presley, et al. (1997). "Golgi tubule traffic and the effects of brefeldin A visualized in living cells." J Cell Biol **139**(5): 1137-55.

Serafini, T., L. Orci, et al. (1991). "ADP-ribosylation factor is a subunit of the coat of Golgi-derived COP-coated vesicles: a novel role for a GTP-binding protein." Cell **67**(2): 239-53.

Sheetz, M. P. and S. J. Singer (1974). "Biological membranes as bilayer couples. A molecular mechanism of drug-erythrocyte interactions." Proc Natl Acad Sci U S A **71**(11): 4457-61.

Shibata, Y., C. Voss, et al. (2008). "The reticulon and DP1/Yop1p proteins form immobile oligomers in the tubular endoplasmic reticulum." J Biol Chem **283**(27): 18892-904.

Shindou, H., D. Hishikawa, et al. (2009). "Recent progress on acyl CoA: lysophospholipid acyltransferase research." J Lipid Res **50 Suppl**: S46-51.

Shindou, H. and T. Shimizu (2009). "Acyl-CoA:lysophospholipid acyltransferases." J Biol Chem **284**(1): 1-5.

Sholl, D. A. (1953). "Dendritic organization in the neurons of the visual and motor cortices of the cat." J Anat **87**(4): 387-406.

Short, B., A. Haas, et al. (2005). "Golgins and GTPases, giving identity and structure to the Golgi apparatus." Biochim Biophys Acta **1744**(3): 383-95.

Silletta, M. G., M. Di Girolamo, et al. (1997). "Possible role of BARS-50, a substrate of brefeldin A-dependent mono-ADP-ribosylation, in intracellular transport." Adv Exp Med Biol **419**: 321-30.

Spang, A. (2008). "The life cycle of a transport vesicle." Cell Mol Life Sci **65**(18): 2781-9.

Stone, S. J., M. C. Levin, et al. (2006). "Membrane topology and identification of key functional amino acid residues of murine acyl-CoA:diacylglycerol acyltransferase-2." J Biol Chem **281**(52): 40273-82.

Stornaiuolo, M., L. V. Lotti, et al. (2003). "KDEL and KKXX retrieval signals appended to the same reporter protein determine different trafficking between endoplasmic reticulum, intermediate compartment, and Golgi complex." Mol Biol Cell **14**(3): 889-902.

Sudhof, T. C. and J. E. Rothman (2009). "Membrane fusion: grappling with SNARE and SM proteins." Science **323**(5913): 474-7.

Terasaki, M., L. B. Chen, et al. (1986). "Microtubules and the endoplasmic reticulum are highly interdependent structures." J Cell Biol **103**(4): 1557-68.

Terasaki, M., J. Song, et al. (1984). "Localization of endoplasmic reticulum in living and glutaraldehyde-fixed cells with fluorescent dyes." Cell **38**(1): 101-8.

Thyberg, J. and S. Moskalewski (1985). "Microtubules and the organization of the Golgi complex." Exp Cell Res **159**(1): 1-16.

Vergnes, L., A. P. Beigneux, et al. (2006). "Agpat6 deficiency causes subdermal lipodystrophy and resistance to obesity." J Lipid Res **47**(4): 745-54.

Weigert, R., M. G. Silletta, et al. (1999). "CtBP/BARS induces fission of Golgi membranes by acylating lysophosphatidic acid." Nature **402**(6760): 429-33.

Yamashita, A., N. Kawagishi, et al. (2001). "ATP-independent fatty acyl-coenzyme A synthesis from phospholipid: coenzyme A-dependent transacylation activity toward lysophosphatidic acid catalyzed by acyl-coenzyme A:lysophosphatidic acid acyltransferase." J Biol Chem **276**(29): 26745-52.

Yamashita, A., H. Nakanishi, et al. (2007). "Topology of acyltransferase motifs and substrate specificity and accessibility in 1-acyl-sn-glycero-3-phosphate acyltransferase 1." Biochim Biophys Acta **1771**(9): 1202-15.

Yang, J. S., H. Gad, et al. (2008). "A role for phosphatidic acid in COPI vesicle fission yields insights into Golgi maintenance." Nat Cell Biol **10**(10): 1146-53.

Yang, J. S., S. Y. Lee, et al. (2005). "A role for BARS at the fission step of COPI vesicle formation from Golgi membrane." Embo J **24**(23): 4133-43.

Yeaman, C., M. I. Ayala, et al. (2004). "Protein kinase D regulates basolateral membrane protein exit from trans-Golgi network." Nat Cell Biol **6**(2): 106-12.

Yuki, K., H. Shindou, et al. (2009). "Characterization of mouse lysophosphatidic acid acyltransferase 3: an enzyme with dual functions in the testis." J Lipid Res **50**(5): 860-9.